Molecular and Biological Studies of Fast and Slow Killing Granuloviruses

Sally Louise Wormleaton BSc

A Thesis Submitted for the Degree of

Doctor of Philosophy

at the

University of Warwick

Department of Entomological Sciences
Horticulture Research International
Wellesbourne

May 2000

Table of Contents

List of Figures

List of tables

Acknowledgements

I would like to thank my supervisor Dr. Doreen Winstanley for her endless help, constant support and sound advice during the course of my studies. I would also like to thank my University supervisor, Dr. Keith Leppard who gave readily of his valuable time.

I would particularly like to thank Howard for his patience and support throughout the last three years. I would also like to thank my Mum, Dad and sister for their never ending encouragement.

I would like to thank John Fenlon for help with statistical analysis, Richard Sampson for photography, Carol Everand for preparation of EM samples and Pauline Goldstraw for rearing of larvae used in this study.

I would like to acknowledge staff and management of Horticulture Research International for providing me with such excellent opportunities and facilities for completing this work.

I would also like to acknowledge the BBSRC for their financial support.

Declaration

I declare that all the work presented in this thesis is my own, performed under the supervision of Dr. D. Winstanley. The contribution of others has been specifically acknowledged. All sources of information used have been referenced. None of the information contained in this thesis has been previously submitted for any other degree.

Summary

The aim of this project was to examine the relationship between granuloviruses (GVs) and factors or genes governing host range, speed of kill and tissue tropism. Hybridisations performed between "fast-killing" (fast) and "slow-killing" (slow) GVs showed that fast GVs appeared to be closely related, as did slow GVs. Fast GVs showed low DNA similarity and only partial collinearity to slow GVs. The exception was *Adoxophyes orana* GV (AoGV), which is a slow GV but showed relatively high DNA similarity and collinearity to fast GVs. This implied that the differences in speed of kill were not necessarily due to the large variations within the genome between fast and slow GVs. Dose and time mortality studies were performed using AoGV and the genome was physically mapped. This would allow easier access to areas of interest on the genome such as regions that did not hybridise to other GVs, that could be unique to AoGV, and would allow the initiation of a sequencing project of the whole genome. The granulin-containing area of the genome was sequenced for comparisons to other GVs. From these data, it is proposed that the relatedness of GVs is dependent on the family of Lepidoptera they infect rather than the tissue tropism of the virus or its speed of kill.

An attempt to expand the host range of *Cryptophlebia leucotreta* GV (ClGV), to include *Cydia pomonella*, was undertaken by recombination with *Cydia pomonella* GV (CpGV). In one experiment ClGV replication was rescued in *C. pomonella* cells. This was either by complementation of the genome by CpGV or recombination with a gene or factor involving host range and will require further studies to identify the gene/factor responsible.

Any recombinant GVs need to be assessed thoroughly for possible expansion of host range to non-target insects. Therefore, transfer vectors were constructed for the production of recombinant GVs containing a reporter gene (*lac*Z). A recombinant CpGV expressing β-galactosidase was produced and will need to be cloned before further work is performed. The course of CpGV infection in hosts differing in permissivity to CpGV could also be studied using these recombinant viruses.

Another part of this research investigated speed of kill. It involved the study of a 2.45 kbp region in CpGV-M1, which was not present in the genotype CpGV-R3. CpGV-M1 is faster killing than CpGV-R3. A recombinant CpGV-R3 that contained this extra region from CpGV-M1 was produced and was compared to CpGV-R3 and CpGV-M1 by bioassay to assess its speed of kill. The speed of kill was not increased indicating that this region was not responsible. However, the study revealed some putative origins of replication of the CpGV genome.

List of Abbreviations

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	**X-gal**
Ethylenediaminetetra-acetic acid	**EDTA**
Luria Bertani	**LB**
Sodium dodecyl sulfate	**SDS**
Tris-acetate-EDTA buffer	**TAE**
Tris-EDTA buffer	**TE**
Occlusion body	**OB**
Granulovirus	**GV**
Nucleopolyhedrovirus	**NPV**
Multiple NPV	**MNPV**
Single NPV	**SNPV**
Days post infection	**d p.i.**
Hours post infection	**h p.i.**
Median lethal dose	**LD_{50}**
Median survival time	**ST_{50}**
Median development time	**DT_{50}**
Base pair	**bp**
Kilobase pair	**kbp**
Juvenile hormone	**JH**
Juvenile hormone esterase	**JHE**
Ecdysteroid UDP-glucosyl transferase	**EGT**
Phenylthiocarbamide	**PTC**
Open reading frame	**ORF**
Cydia pomonella GV	**CpGV**
Cryptophlebia leucotreta GV	**ClGV**
Adoxophyes orana GV	**AoGV**
Lacanobia oleracea GV	**LoGV**
Pieris (Artogeia) rapae GV	**PrGV**
Plutella xylostella GV	**PxGV**
Phthorimaea operculella GV	**PoGV**
Spodoptera littoralis GV	**SlGV**
Choristoneura fumiferana GV	**CfGV**
Trichoplusia ni GV	**TnGV**
Xestia-c nigrum GV	**XcGV**
Epinotia aporema GV	**EaGV**
Autographa californica MNPV	**AcMNPV**
Lymantria dispar NPV	**LdNPV**
Orgyia pseudotsugata NPV	**OpNPV**

CHAPTER 1

Introduction

1.1 Classification of baculoviruses

The *Baculoviridae* are a family of large double stranded DNA viruses of between 90-200 kilobase pairs (kbp) and have been extensively reviewed by Miller (1997) and Hunter *et al.* (1998). Baculoviruses infect the larval stage of arthropods mainly from the class Insecta (Murphy *et al.*, 1995). The covalently closed circular DNA genome is packaged in a nucleocapsid which is enveloped and embedded in a proteinaceous occlusion body (OB) (Joel Funk *et al.*, 1997). The OB protects the virion from adverse conditions such as UV light and desiccation (Vialard *et al.*, 1995).

There are two genera of baculovirus, the nucleopolyhedrovirus (NPV) and the granulovirus (GV) (Murphy *et al.*, 1995). The NPVs have large polyhedra shaped OBs with diameters of between 1 to 5 μm with single or multiple virions embedded (Federici, 1997). The GVs have small oval shaped OBs which average about 150 nm x 300-600 nm normally containing a single virion (Federici, 1997). As a group, NPVs have a wider host range than GVs, being isolated mainly from the order Lepidoptera but also Diptera, Hymenoptera and others (Murphy *et al.*, 1995). To date, GVs have only been isolated from Lepidopteran larvae (Murphy *et al.*, 1995). There are two types of NPV based on whether the nucleocapsid is singly enveloped (SNPV) or multiple nucleocapsids are enveloped within a single virion (MNPV) (Murphy *et al.*, 1995). However, there is no longer a subdivision as this difference does not appear to be very significant taxonomically (Hunter *et al.*, 1998).

1.2 Importance of baculoviruses

1.2.1 Biocontrol agents

Baculoviruses cause disease in many important pests of agricultural and horticultural crops and have the potential to be used as biocontrol agents in place of chemical insecticides (Black *et al.*, 1997). The use of baculoviruses as biological control agents is reviewed in Cory and Bishop (1995) and Moscardi (1999). The advantages of using baculoviruses instead of chemical insecticides are that baculoviruses are much more specific than chemical insecticides, therefore beneficial or non-target insects are not affected (Black *et al.*, 1997). Also, baculoviruses occur naturally in the environment, and are therefore considered more environmentally friendly than

chemical insecticides. Disadvantages are that baculoviruses are slower killing than chemical insecticides taking several days to kill, resulting in continued crop damage. The narrow host range of baculoviruses can be a disadvantage if the control of more than one species of pest per crop is required. Another problem with controlling pests is that in some cases the larvae, shortly after hatching, will enter the fruit. This means the timing of the application has to be precise, in order to infect and preferably kill the larvae before entry into the fruit.

Baculoviruses are registered and used as biocontrol agents in several countries. Several NPVs are used, with the most successful being *Anticarsia gemmatalis* NPV, where over one million hectares per annum are sprayed for control of the soybean caterpillar in Brazil (Moscardi, 1999). The GVs which are currently registered and used are *Adoxophyes orana* GV (AoGV) for control of apple pests in Switzerland, *Cydia pomonella* GV (CpGV) for control of apple and pear pests in Germany, France, USA and Russia and *Phthorimaea operculella* GV (PoGV) for control of potato pests in Peru, Egypt and Tunisia (Moscardi, 1999).

The insecticidal properties of baculoviruses can be increased by genetic engineering. The development and use of recombinant viruses has been reviewed in Bonning and Hammock (1996), Richards *et al.* (1998), Possee *et al.* (1997), Miller (1995), Hawtin *et al.* (1992) and Wood and Granados (1991). Several strategies have been undertaken to increase the efficacy of baculoviruses. These generally involve deletion of a non-essential gene and insertion of a gene with insecticidal properties under the control of the hyperexpressed polyhedrin, granulin or *p10* promoter. It has been possible to genetically manipulate a few NPVs for which virus-permissive cell lines are available. Most research has focused on AcMPNV, since this can be propagated easily in cell culture and has received most attention because of its use as an expression vector.

Gene deletions alone can increase the insecticidal properties of some baculoviruses. The *egt* gene encodes an enzyme, ecdysteroid UDP-glucosyl transferase (EGT) that inactivates the host moulting hormone, ecdysone. The gene was initially discovered in *Autographa californica* NPV (AcMNPV) (O'Reilly and Miller, 1989, 1990). When larvae go through larval-larval moults and larval-pupal moults they cease

feeding. This gene functions to increase the yield of virus produced by inhibiting moulting thereby allowing the larvae to continue feeding (O'Reilly and Miller, 1991). The more that the larvae feed, the larger they get and more virus is produced (O'Reilly and Miller, 1991). An AcMNPV EGT$^-$ recombinant virus was made (O'Reilly and Miller, 1991) and it was observed that the EGT$^-$ infected larvae died up to 30% faster than those infected with the wild-type virus (O'Reilly and Miller, 1991). The *egt* gene is a non-essential viral gene and therefore, is an ideal gene to remove and replace with an insecticidal gene to increase the speed of kill of the virus even further. The effect of deleting the *egt* gene may vary depending on the particular virus, the host or the instar infected and therefore may not have effects as dramatic as the AcMNPV EGT$^-$ recombinant (D. Winstanley, personal communication).

Two types of gene have been inserted into baculovirus genomes in an attempt to increase the speed of kill. The first type encodes insect hormones or enzymes. Over-expressing insect hormones may adversely affect the physiology or development of the larvae. Although genes such as those encoding diuretic hormone (Maeda, 1989*b*) prothoracicotropic hormone (PTTH) (O'Reilly *et al.*, 1995) and eclosion hormone (Eldridge *et al.*, 1992) have been inserted into baculoviruses, they were not found to increase the speed of kill significantly. However, Gopalakrishnan *et al.* (1995) showed that the insertion of an insect chitinase gene into AcMNPV resulted in a 20% increase in speed of kill. Also, three AcMNPV recombinants expressing the enzyme juvenile hormone esterase (JHE) have been constructed in which site directed mutagenesis had been used to alter amino acids. The first altered amino acids that were thought to be involved in the degradation process of JHE (Ward *et al.*, 1992). The second altered amino acids within the catalytic domain of JHE (Bonning *et al.*, 1995) and the third altered amino acids resulting in decreased efficiency of lysosomal targeting in pericardial cells (Bonning *et al.*, 1999). These recombinant viruses were found to show significantly reduced lethal times and feeding damage compared to the wild-type virus.

The second type of gene to be inserted encodes insect-specific toxins. The AaIT gene which encodes the insect-specific venom component of the North African scorpion *Androctonus australis* (Zlotkin *et al.*, 1971; Darbon *et al.*, 1982) has been inserted into the AcMNPV genome (Maeda *et al.*, 1991) and shows huge potential for the use

as a biocontrol agent. The TxP-I toxin, which is a venom component of the predatory straw itch mite, *Pyemotes tritici* (Tomalski *et al.*, 1988*a*, 1989), shows similar promise.

1.2.2 Expression vectors

Baculoviruses are used widely as expression vectors and this topic has been reviewed in Maeda (1989*a*), King and Possee (1992), O'Reilly *et al.* (1992) and Possee, (1997). These expression vectors principally exploit the hyperexpressed polyhedrin or *p10* promoter of NPVs (Smith *et al.*, 1983; Vlak *et al.*, 1990) but also promoters of early genes such as the *ie1* promoter (Jarvis *et al.*, 1996). Extensive research and development on the baculovirus expression system, based on AcMNPV have resulted in a relatively easy to use procedure to produce proteins in eukaryotic cells. This has advantages since biologically active forms of complex polypeptides are sometimes difficult to produce using expression vectors in prokaryotic cells (Maeda, 1989). Furthermore, certain post-translational modifications do not occur in prokaryotic cells (Maeda, 1989). To use the system, the foreign gene of interest is placed under the control of the baculovirus promoter within a plasmid transfer vector, which then recombines with a baculovirus (usually AcMNPV) in cultured cell lines. The resulting recombinant virus will then produce the foreign mRNA and protein, which will be modified in the eukaryotic cells.

1.3 Baculovirus replication *in vivo*

1.3.1 NPVs

There are two phenotypes of baculovirus, the budded virus and the occlusion-derived virus (Joel Funk *et al.*, 1997). The replication of baculoviruses is described in Miller (1997), Blissard and Rohrmann (1990), Vialard *et al.* (1995) and King and Possee (1992) and is summarised below and in Figure 1.1. The occluded virus is the form present in the environment. Occlusion bodies (OBs) are ingested and dissolve in the alkaline midgut of the larvae. The virions are released and attach and fuse to the microvilli of midgut cells. The nucleocapsid is then transported to the nucleus where it is uncoated. Transcription and replication then take place within the nucleus. Nucleocapsids are formed within the nucleus and bud through the nuclear membrane acquiring a lipid envelope. This envelope is lost in the cytoplasm. The budded virions then acquire an envelope from the plasma membrane of the cell as they leave

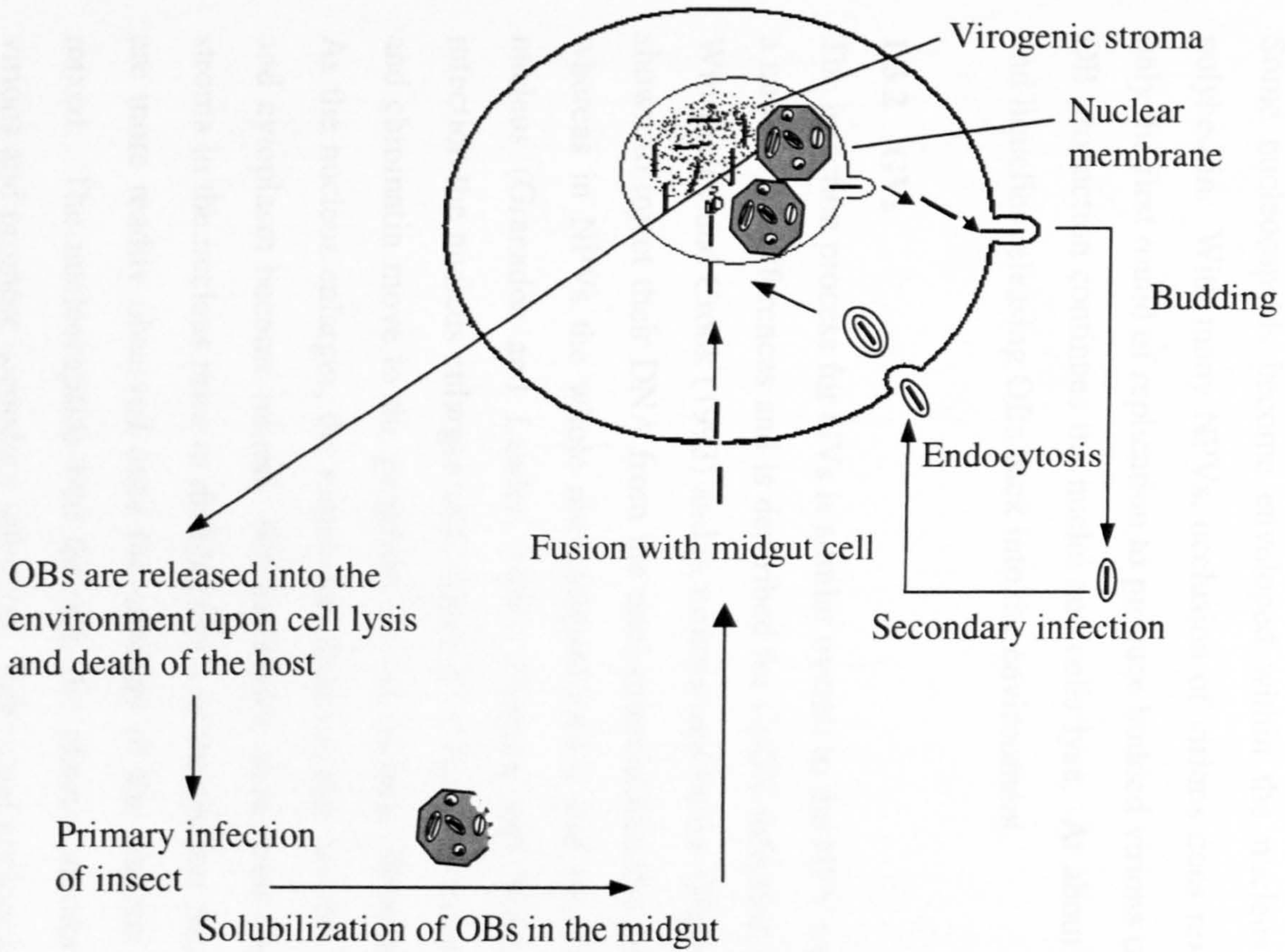


Figure 1.1
Life cycle of a nucleopolyhedrovirus in an infected insect cell. OBs are ingested by the insect host and solubilized in the alkaline midgut of the host. The released virions attach and fuse with midgut cells. The nucleocapsid is targeted to the nucleus where the viral DNA is uncoated. Transcription and replication occurs in the nucleus and nucleocapsids are formed within a dense virogenic stroma. Some nucleocapsids bud through the nuclear membrane and are transported to the plasma membrane but lose the nuclear derived envelope. These nucleocapsids bud through the plasma membrane acquiring the budded virus specific envelope. The budded virions then initiate secondary infection to adjacent cells. Occlusion occurs within the nucleus. Upon cell lysis and insect death the OBs are released into the environment.

the cell. This envelope contains a viral-encoded envelope glycoprotein named *gp64* in AcMNPV infection (Whitford *et al.*, 1989; Monsma *et al.*, 1996). LdMNPV does not encode a homologue of *gp64* but does contain ORF 130 which encodes a protein indicative of transmembrane receptor-like proteins (Kuzio *et al.*, 1999). It is proposed that in AcMNPV infection budded virions are transported throughout the host via the tracheal matrix and the haemolymph where they will infect further cells producing a second round of replication (Engelhard *et al.*, 1994). Cytopathic effects observed during this phase include enlargement of the nucleus and development of virogenic stroma within the nucleus, which is thought to be the site of nucleocapsid assembly. Some nucleocapsids become enveloped within the nucleus and later occluded in polyhedrin. With many NPVs, occlusion of virions does not occur in midgut cells, only the first round of replication to produce budded virions occurs in these cells. As OB production continues the nuclei and cells lyse. At about this time the larva dies and liquefies releasing OBs back into the environment.

1.3.2 GVs

The infection process for GVs is similar overall to the NPV infection process but with a number of differences and is described for CpGV infection in *C. pomonella* cells in Winstanley and Crook (1993) and is summarised below (Figure 1.2). GVs have been shown to inject their DNA from the nucleocapsid into the nucleus (Summers, 1971) whereas in NPVs the whole nucleocapsid enters and the DNA is uncoated in the nucleus (Granados and Lawler, 1981; Charlton and Volkman, 1993). Early in infection the nucleus enlarges and 'clearing' of the nucleus occurs where the nucleoli and chromatin move to the periphery of the nucleus (Winstanley and Crook, 1993). As the nucleus enlarges, the membrane disintegrates and the contents of the nucleus and cytoplasm become mixed. Nucleocapsids have been observed within virogenic stroma in the nucleus prior to disintegration of the nuclear membrane. However, they are more readily observed once the contents of the nucleus and cytoplasm become mixed. The nucleocapsids bud through the plasma membrane to become budded virions and promote secondary infection. Individual nucleocapsids are enveloped and occluded within masses of granulin throughout the cell. As the number of OBs increase the cell greatly enlarges, eventually containing thousands of granules. These cells will eventually lyse. When the larvae die, the remaining cells lyse and the OBs are released back into the environment.

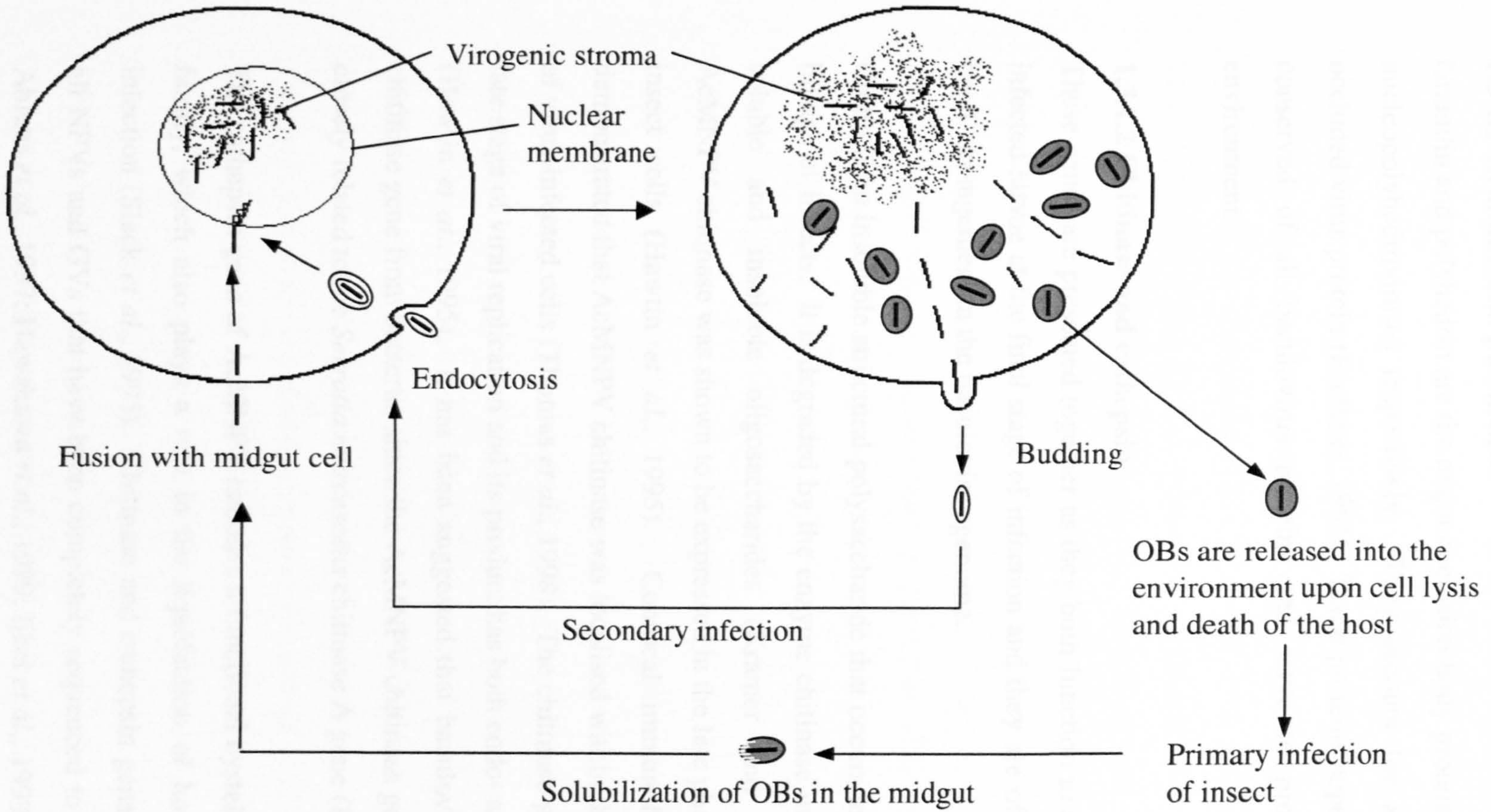


Figure 1.2
Life cycle of a granulovirus in an infected insect cell. OBs are ingested by the insect host and solubilized in the alkaline midgut of the host. The released virions attach and fuse with midgut cells. The nucleocapsid is targeted to the nuclear membrane where the viral DNA is injected into the nucleus. Nucleocapsids begin to form within a virogenic stroma. The nuclear membrane breaks down and occlusion of virions occurs throughout the cell. Nucleocapsid production continues within the virogenic stroma. Some progeny nucleocapsids bud through the plasma membrane acquiring the budded virus specific envelope. The budded virions then initiate secondary infection to adjacent cells. Upon cell lysis and insect death the OBs are released into the environment.

1.3A Conserved baculovirus genes

1.3A.1 Granulin and polyhedrin

Granulin and polyhedrin are the major occlusion body proteins for granuloviruses and nucleopolyhedroviruses respectively. They account for about 95% of the total occluded virus protein (Faulkner, 1981). These proteins appear to be the most highly conserved of all baculovirus proteins and serve to protect the virion in the environment.

1.3A.2 Chitinase and cathepsin

These genes are presented together as they both function to aid in the liquefaction of infected larvae at the final stage of infection and they are often located in a 'head to head' arrangement in the baculovirus genome.

Chitin is an insoluble structural polysaccharide that occurs in the exoskeleton and gut linings of insects. It is degraded by the enzyme chitinase into low molecular weight soluble and insoluble oligosaccharides (Kramer and Muthukrishnan, 1997). AcMNPV chitinase was shown to be expressed in the late phase of virus replication in insect cells (Hawtin *et al.*, 1995). Confocal immunofluorescence microscopy demonstrated that AcMNPV chitinase was localised within the endoplasmic reticulum of virus-infected cells (Thomas *et al.*, 1998). The chitinase gene was expressed in the late stage of viral replication and its product has both endo- and exo- chitinase activity (Hawtin *et al.*, 1995). It has been suggested that baculoviruses have acquired the chitinase gene from bacteria, since the AcMNPV chitinase gene was found to be most closely related to the *Serratia marcescens* chitinase A gene (Hawtin *et al.*, 1995).

The cathepsin gene of AcMNPV encodes a functional cysteine protease of the papain family, which also plays a role in the liquefaction of host tissues during a viral infection (Slack *et al.*, 1995). Chitinase and cathepsin genes have been identified in all NPVs and GVs that have been completely sequenced to date (Ayres *et al.*, 1994; Ahrens *et al.*, 1997; Hawakawa *et al.*, 1999; Ijkel *et al.*, 1999; Kuzio *et al.*, 1999) with the exception of *Plutella xylostella* GV (Hashimoto *et al.*, 1999). Deletion of either the cathepsin or chitinase genes resulted in the failure of AcMNPV to cause liquefaction of the host (Hawtin *et al.*, 1997). This indicated that these proteins function together to promote degradation of the host tissues at the end of the infection

process (Hawtin *et al.*, 1997). It appears that baculoviruses encode these enzymes to aid in the breakdown of insect tissues at the end of infection to release OBs into the environment and aid in their horizontal spread.

1.3A.3 Superoxide dismutases (SODs)

Superoxide dismutase is a conserved gene of baculoviruses. SODs catalyse the dismutation of the superoxide radical O_2- into H_2O_2 and O_2 (Pardini, 1995). The superoxide radical and its metabolites cause damage to most cellular constituents. Therefore SODs provide some protection against oxygen toxicity (Pardini, 1995). However, the function of SODs in the baculovirus genome is unknown. The gene has been deleted in AcMNPV with no deleterious effect (Tomalski *et al.*, 1991). Also host SODs are abundant in infected insect cells and therefore a baculovirus copy would not be required. Some studies suggest that SOD may serve to protect OBs from superoxide radicals generated by exposure to sunlight in the environment (Tomalski *et al.*, 1991).

1.3A.4 Ubiquitin

Ubiquitins are small proteins which are abundantly present in eukaryotic cells. They are thought to be involved in an array of basic cellular processes and have been strongly implicated in protein degradation (Doherty and Mayer, 1992; Finley and Chau, 1991). Ubiquitin has been found in all baculoviruses sequenced to date. It is a highly conserved protein and with the exception of polyhedrin and granulin it is the most highly conserved gene in the baculovirus genome. Ubiquitin appears to be nonessential for viral replication (Fraser *et al.*, 1995). However, the yield of budded virus was five to ten fold less in a mutant with a frameshift mutation in the ubiquitin gene of AcMNPV (Reilly and Guarino, 1996). This suggests that it may be involved in the formation of progeny budded virions (Reilly and Guarino, 1996).

1.3A.5 p74

The p74 protein appears to be expressed at low levels late in infection. A partial *p74* deletion mutant of AcMNPV was capable of replicating in cultured cells but the resulting occluded virus was unable to infect *Trichoplusia ni* larvae *per os* (Kuzio *et al.*, 1989). Therefore, p74 appears to be essential for production of infectious occlusion bodies (Kuzio *et al.*, 1989).

1.4 Baculovirus replication *in vitro*

Several NPVs have been found to replicate well in cell culture with many cell lines existing that support their replication, some of which are commercially available. In contrast, there are only a few laboratories world-wide that have managed to sustain a GV permissive cell line. Winstanley *et al.* (1993) reported a *Cydia pomonella* embryonic cell line permissive for CpGV. This was the cell line used in these studies. TnGV was reported to infect *Trichoplusia ni* cells but their permissive character was not maintained (Granados *et al.*, 1986). However, complete viral replication of *Phthorimaea operculella* GV has been obtained in a *P. operculella* cell line (Léry *et al.*, 1997; 1998). The ability of GV and SNPV to replicate in culture appears to be dependent on the rate of cell growth, with permissive cells being the slow growing ones which were attached as monolayers (Naser *et al.*, 1984).

1.5 Baculovirus gene expression and replication

The gene expression of baculoviruses is sequential and co-ordinated. It is split essentially into two stages. Early gene expression, which precedes DNA replication and late/very late gene expression, which occurs after the onset of DNA replication. The successive stages of viral gene expression depend on the previous gene products. Early and late genes are distributed randomly along the genome and transcribed from both strands of DNA (Friesen, 1997).

1.5.1 Early gene expression

Early genes are defined as genes transcribed prior to the initiation of virus DNA replication. Early genes are thought to be transcribed using the host RNA polymerase II. This has been supported by the fact that naked DNA devoid of any virion components is infectious in cell culture (Freisen, 1997) and early transcription is blocked by the RNA polymerase II inhibitor α-amanitin (Huh and Weaver, 1990; Grula *et al.*, 1981). Therefore, promoters of baculovirus early genes tend to mimic promoters of eukaryotic genes. They characteristically have a TATA box with a RNA transcription start site 25-31 bp downstream, which usually contains the consensus CAGT, although it is the CA dinucleotide which appears to be most important (Kogan

et al., 1995; Pullen and Friesen, 1995). Other early gene promoter sequences have also been identified such as the sequence CGTGC that is positioned as the RNA start site of AcMNPV genes such as *p47*, *p143* and *dnapol* (Tomalski *et al.*, 1988*b*; Ohresser *et al.*, 1995). It is likely that more early gene promoter sequences will be discovered with the sequencing of more baculoviruses. Transcription occurs as soon as the viral DNA is uncoated in the nucleus. Early genes function to accelerate replicative events and to prepare the host cell for virus multiplication (Friesen, 1997). Early genes can also directly regulate the host to optimise virus replication. For example, the *egt* gene is an early gene in AcMNPV and inhibits moulting, ultimately increasing virus replication (O'Reilly and Miller, 1989, 1990). Also certain anti-apoptotic genes are expressed early in infection. These genes prevent the anti-viral host defence of apoptosis from destroying infected cells and allow the infection process to proceed.

1.5.2 DNA replication

DNA replication initiates a cascade of gene expression that ultimately results in the production of progeny virus and is reviewed in Kool *et al.* (1995). Baculovirus gene products are required for DNA replication therefore it is dependent on early viral gene expression. Baculoviruses replicate using a novel DNA polymerase distinct from host cell polymerases (Mikhailov *et al.*, 1986; Wang and Kelly; 1983; Miller *et al.*, 1981). All baculovirus genomes sequenced to date have been shown to have regions within their genome called homologous repeats (*hrs*) (Ayres *et al.*, 1994; Ahrens *et al.*, 1997; Kuzio *et al.*, 1999; Hayakawa *et al.*, 1999; Ijkel *et al.*, 1999; Hashimoto *et al.*, 1999). These regions are usually spaced out around the genome and have imperfect palindromes or direct repeats that are repeated several times. These elements have been shown to act as origins of DNA replication as well as enhancers of early gene promoters (Guarino *et al.*, 1986; Guarino and Summers, 1986; Leisy and Rohrmann, 1993; Kool *et al.*, 1994*a,b*). The viral DNA is thought to replicate via a rolling-circle mechanism since plasmids containing *hrs* replicated into high-molecular-weight concatemers in virus-infected cells (Leisy and Rohrmann, 1993) and multimers of viral DNA have been detected in virus-infected cells (Oppenheimer and Volkman, 1997).

1.5.3 Late and very late gene expression

The late stage of baculovirus infection is defined as the events occurring after the onset of DNA replication (Lu and Miller, 1997). Late and very late genes are transcribed by a α-amanitin resistant virus-induced RNA polymerase that recognises the motif (A/T/G)TAAG (Beniya *et al.*, 1996; Yang *et al.*, 1991; Huh and Weaver, 1990; Fuchs *et al.*, 1983; Grula and Weaver, 1981; Grula *et al.*, 1981). This core element appears to function as both a promoter and mRNA start site. Structural proteins as well as budded virions are produced during the late phase. The very late stage involves the expression of the occlusion body proteins, granulin or polyhedrin. Also the gene *p10* in AcMNPV infection is transcribed abundantly in the very late phase of infection (van Oers and Vlak, 1997). This gene product aggregates into fibrillar structures and is thought to be involved in rupture of the nucleus in the late stages of infection and in polyhedrin morphogenesis (van Oers and Vlak, 1997). Homologues of *p10* usually have low sequence similarity and definitive homologues in GVs are yet to be discovered although possible homologues with low amino acid identities to structural domains of P10 have been identified in PxGV and XcGV (Hashimoto *et al.*, 1999; Hayakawa *et al.*, 1999). The distinction between late and very late genes is the addition of an extra promoter element between TAAG and the ATG translation start site. Very late gene expression also relies on a late gene product called very late expression factor-1 (VLF-1) (McLachlin and Miller, 1994). This gene product has been found to interact with the additional promoter element (Yang and Miller, 1999). A transient expression assay was used to determine genes required for late and very late expression of AcMNPV (Todd *et al.*, 1995; Lu and Miller, 1995). Eighteen AcMNPV genes were required which are called late expression factors (*lef*s) (Todd *et al.*, 1995). These *lef*s are involved in viral DNA replication and gene expression. The particular *lef*s required can differ in different host cells and *lef*s in other NPVs and GVs will probably be found to differ slightly.

1.6 Host range of baculoviruses

Baculoviruses have a relatively narrow host range, usually infecting only one host or a few closely related hosts (Gröner, 1986). AcMNPV has the widest host range of any baculovirus known to date, infecting at least 33 species of lepidopteran larvae in 10

families (Gröner, 1986). NPVs generally have a broader host range than GVs. The molecular basis of baculovirus host range is an area which is being studied extensively in NPVs but due to the lack of susceptible cell lines has not received much attention in GVs. However, the studies on NPVs have given an insight into some of the mechanisms and controlling factors involved in determination of host range.

Several NPV genes have been identified that have been shown to affect host range determination. AcMNPV is unable to productively infect Ld652Y cells; a cell line derived from *Lymantria dispar* (Guzo *et al.*, 1992; Morris and Miller, 1992). The block is thought to be at the translation level (Guzo *et al.*, 1992; Morris and Miller, 1992). However, when AcMNPV infected Ld652Y cells are superinfected with LdMNPV, normal AcMNPV replication occurs (McClintock and Dougherty, 1987). Therefore, LdMNPV was thought to be complementing the defect in expression from the AcMNPV genome. Ld652Y cells were cotransfected with AcMNPV and overlapping cosmid clones of LdMNPV (Thiem *et al.*, 1996). Cosmids that allowed the production of OBs were subcloned and cotransfected again with AcMNPV. Using this complementation assay the specific area responsible for AcMNPV infection was mapped. It was found to be a novel gene named host range factor-1 (*hrf-1*) (Thiem *et al.*, 1996). A recombinant AcMNPV containing this gene was also able to infect *L. dispar* larvae (Chen *et al.*, 1998). Therefore the host range of AcMNPV had been expanded to include LdMNPV. A mechanism has subsequently been suggested of translation arrest involving defective or depleted tRNA species in AcMNPV infected Ld652Y cells (Mazzacano *et al.*, 1999).

AcMNPV and BmNPV have a very high DNA homology but do not have overlapping host ranges (Miller and Lu, 1997). AcMNPV cannot infect BmN cells, a cell line derived from *Bombyx mori* that supports BmNPV infection, and BmNPV cannot infect SF-9 cells, a cell line derived from *Spodoptera frugiperda* that supports AcMNPV infection (Kondo and Maeda, 1991; Croizier *et al.*, 1994). A recombinant AcMNPV generated by cotransfection of AcMNPV and BmNPV was found to be able to infect BmN cells (Kondo and Maeda, 1991; Croizier *et al.*, 1994). The area responsible was first narrowed down to a 0.6 kbp DNA fragment of BmNPV (Maeda *et al.*, 1993). However, the AcMNPV recombinant resulted in an abortive infection

in Sf-21 cells (another cell line derived from *S. frugiperda* that supports AcMNPV infection) when they were infected with a low multiplicity of infection (m.o.i.), but not when infected with a high m.o.i. (Kamita and Maeda, 1996). The infectivity was also reduced in *S. frugiperda* larvae (Kamita and Maeda, 1996). This suggested that the activity of p143 had been reduced in the recombinant, therefore more p143 was required for a productive infection (Kamita and Maeda, 1996). It is also interesting to note that the AcMNPV p143 protein appeared to have a cytotoxic affect on the BmN cells, which was suggested to be the result of a cellular anti-viral defence mechanism (Kamita and Maeda, 1993). This effect disappeared when the cells were infected with the recombinant AcMNPV, this was presumed to be due to a decrease in p143 cytotoxicity (Kamita and Maeda, 1996). Therefore it has been suggested that the level of p143 activity can be important in determining host range. (Kamita and Maeda, 1996).

The area responsible for the expanded host range was gradually narrowed down to a single amino acid substitution within the p143 helicase protein of AcMNPV (Kamita and Maeda, 1997). Two amino acid substitutions in the AcMNPV p143 specificity region were sufficient to expand the host range of AcMNPV to *B. mori* larvae (Argaud *et al.*, 1998). Therefore the host range of AcMNPV has been expanded to include *Bombyx mori* cells and larvae.

Genes have also been identified that exert a host cell specific effect. The late expression factor (*lef*) library of AcMNPV consists of 18 *lef*s that allow expression of genes under late and very late promoters in Sf-21 cells (Todd *et al.*, 1995; Lu and Miller, 1995). These same *lef*s did not allow expression of genes under late and very late promoters in TN-368 cells (Lu and Miller, 1995a). Another viral gene was required which was named host cell factor –1 (*hcf-1*) (Ayres *et al.*, 1994; Lu and Miller, 1995). This gene had no homologues on databases. In addition, some of the 18 *lef*s were not required for infection of TN-368 cells; these were *ie-2*, *lef-7* and *p35*. Therefore, different *lef*s may be required for virus replication in different host cells, thus affecting host range (Thiem *et al.*, 1996).

Other genes implicated in host range are the anti-apoptotic genes. The *p35* gene of AcMNPV is essential for preventing apoptosis and therefore permitting productive

infection of AcMNPV in Sf-21 cells or *S. frugiperda* larvae. As mentioned above, it is not required for infection of TN-368 cells or *T. ni* larvae (Lu and Miller, 1995). Certain *iap* (inhibitor of apoptosis) genes are able to compensate for *p35* in AcNMPV infection of Sf-21 cells and are therefore implicated in host range (Crook *et al.*, 1993; Birnbaum *et al.*, 1994).

Therefore, it appears that the host has many lines of defence against viral infection. The ability of the virus to overcome these appears in some cases to be dependent on the substitution or addition of a single gene.

1.7 Insertion of reporter genes into baculoviruses

An accurate knowledge of the host range of wild-type and genetically modified baculoviruses is a prerequisite to its use in the field as a biocontrol agent and to assess the environmental risks associated with genetically engineered baculovirus pesticides. Symptomology studies have been widely used but these may overlook hosts that do not show overt signs of infection. There is a lot of concern over the release of genetically manipulated organisms. Concern over human health, effects on non-target organisms, displacement of natural virus populations and gene exchange between recombinant viruses and other organisms have been reported (Wood and Granados, 1991; Wood and Hughes, 1993). By insertion of reporter genes into recombinant viruses, in depth host range studies can be undertaken which will allow visualisation of infection at low levels in what would otherwise be regarded non-permissive hosts. This approach provides a more realistic insight into the exact host range of recombinant viruses.

It is important to have a greater understanding of the infection process, the mechanism of virus entry and dissemination of GVs within larval tissues. Similar studies have been performed with a few NPVs because of the relative ease of producing the NPV recombinants. A recombinant AcMNPV containing the *lacZ* reporter gene (AcMNPV-*hsp70/lacZ*) was made and used to study resistance in *Helicoverpa zea*, a pest non-permissive to AcMNPV (Washburn *et al.*, 1996). It was found that the *H. zea* larvae were actually highly susceptible to the virus infection but that the infected cells were encapsulated and cleared by haemocytes (Washburn *et al.*, 1996). This suggested that

the immune response of the insect also plays a role in host range (Washburn *et al.*, 1996).

AcMNPV was found to express genes under early viral as well as host promoters in a variety of cell lines from semi-permissive and non-permissive lepidopteran and dipteran cell lines (Morris and Miller, 1992). It therefore appears that entry into the cell is not generally the barrier to infection by this virus. DNA can be presented in an expressible form; therefore the block to infection in most cases occurs after or during early gene expression (Morris and Miller, 1992).

1.8 Fast and slow killing granuloviruses

Nucleopolyhedroviruses predominantly infect the order Lepidoptera, but are also known to infect Hymenoptera and Diptera and possibly other orders of Insecta (Federici, 1997). GVs have only been isolated from Lepidopteran hosts and the International Committee of the Taxonomy of Viruses (ICTV) has acknowledged no formal classification of GVs. Three types of GV have been proposed, based on the tissue tropism of the virus (Federici, 1997). Type 1 granuloviruses, such as *Trichoplusia ni* GV (TnGV) infect only the fat body of the larvae after the virus has passed through the midgut epithelium. These infected larvae tend to take longer to die than other GV or NPV-infected larvae. When fourth instar *T. ni* larvae are infected with TnGV, they typically take 10-14 days to die (Federici, 1997). A possible explanation for this is that important tissues such as the tracheal matrix and the epidermis have not been infected (Federici, 1997). Slow-killing viruses tend to kill in the final instar regardless of the instar in which the larvae were infected. These viruses predominantly infect larvae belonging to the family Noctuidae and tend to have large genomes of over 150 kbp. In this thesis, these GVs will be referred to as 'slow' GVs.

Type 2 GVs, such as *Cydia pomonella* GV (CpGV) are fast-killing, taking only 5-10 days in larvae that are infected in the fourth instar, and have a much broader tissue tropism than slow GVs (Federici, 1997). In this thesis, these GVs will be referred to as 'fast' GVs. The fat body is infected along with the epidermis, malpigian tubules, tracheal matrix and haemocytes as well as many other tissues to a lesser extent. The faster speed of kill could be due to the infection of major body tissues (Federici, 1997).

Fast GVs tend to kill in the instar in which the larvae were infected or within the next instar. These viruses infect larvae from a variety of families of Lepidoptera including Tortricidae, Pieridae, Yponomeutidae, Pyralidae and Gelechiidae. The genomes of fast GVs have a tendency to be smaller than the genomes of slow GVs. They are usually between 100-135 kbp in length.

The third type of granulovirus contains only one virus to date. This is *Harrisina brillians* GV (HbGV) (Federici, 1997). HbGV replicates principally in the midgut epithelium cells of *Harrisina brillians* larvae which is from the family Zygaenidae (Smith *et al.*, 1956). The larvae develop diarrhoea which consists of a discharge containing OBs (Crook, 1991). This can lead to a rapid transmission of infection (Crook, 1991). *H. brillians* larvae take longer to die the earlier in their larval development that they are infected (Smith *et al.*, 1956) which is also typical of slow GVs.

1.9 The codling moth, *Cydia pomonella*, a lepidopteran pest of apples

The codling moth *C. pomonella* (Lepidoptera: Tortricidae) is a pest of economic importance in commercial apple orchards throughout the world. It is also a pest of pears, peaches and walnuts. Damage to the fruit is caused by the larval stage. Moths of *C. pomonella* usually lay their eggs on the leaves of affected trees and after hatching, larvae bore into the flesh of the fruit and tunnel to the core. The larvae feed inside the fruit for all their larval development, usually lasting 21-25 days, before leaving the fruit to overwinter or to pupate (Hill, 1987). Damaged fruit also becomes susceptible to secondary problems such as rotting. Fruit infested with larvae of the codling moth become unmarketable by the commercial grower and are considered inedible by the consumer.

C. pomonella is a pest of temperate and tropical regions but the severity of the pest problem varies with climate. In relatively cool regions such as Northern Europe, the codling moth is usually restricted to one generation per season (Subinprasert and Svensson, 1988) but sometimes in warm springs and summers in the UK it can produce two generations (Glen *et al.*, 1976). There are usually two generations in

Germany (Huber and Dickler, 1977), three in Chile (Gonzalez, 1982) and four in Tunisia (Mussche and Garbous, 1986).

C. pomonella is controlled mainly by the timely application of insecticides. Timing is critical because most damage occurs once the larvae enter the fruit. Therefore, control must occur during the period following their hatching and before entry into the fruit, usually as neonate or second instar larvae. Death of larvae within the first instar results in only superficial damage to the fruit referred to as 'sting damage' (Glen and Clark, 1985).

Broad spectrum insecticides were used to control codling moth but these were also detrimental to beneficial arthropods such as *Typhlodromus pyri* and lead to infestation by secondary pests including the fruit tree red spider mite *Panonychus ulmi* (Collyer, 1953; Solomon, 1982). The advent of insecticide-resistance has led to the use of pheromone mating disruption as an alternative control measure but this technique is costly (Cross, *et al.*, 1999). At a time of growing public concern over pesticide residues in food and the environment, an effective but safe insecticide for controlling *C. pomonella* is needed.

A granulovirus causing mortality to *C. pomonella* larvae was first isolated from dead overwintering larvae in Mexico in 1963 (Tanada, 1964; Thomas and Poinar, 1973). This isolate was named CpGV-M. Several other wild-type isolates were identified subsequently from Russia and England (Cross *et al.*, 1999). Field trials in Northern California in 1966 showed that CpGV-M reduced the number of overwintering larvae by 80 % (Falcon *et al.*, 1968). Field trials in the UK showed that deep fruit damage was reduced by up to 90 % (Glen and Payne, 1984), while in Chile, deep fruit damage was reduced by up to 100% (Ripa, 1982). Three commercial formulations of CpGV are currently available in Europe (Cross *et al.*, 1999). The major advantage of using the virus is reduced environmental hazard by the reduction of insecticide use as well as the preservation of natural enemies for control of secondary pests. The virus is highly virulent against codling moth, particularly neonates which can be killed after ingestion of a single OB. CpGV is also an effective biological control agent for the pea moth, *Cydia nigricana* and the pine shoot moth, *Rhyacionia buoliana* (Huber, 1982). It is also moderately infectious to neonates of the larvae of the false codling

moth, *Cryptophlebia leucotreta.* The major disadvantage in using CpGV for control is that it kills more slowly than conventional insecticides, and if neonate larvae do not ingest virus, considerable damage occurs.

There are three isolates of CpGV known to date. CpGV-M, a Mexican isolate (Tanada, 1964), an English isolate, CpGV-E (Crook *et al.*, 1985) and a Russian isolate, CpGV-R (Harvey and Volkman, 1983; Crook *et al.*, 1985). Only one genotype has been cloned *in vivo* from CpGV-M, this was named CpGV-M1. CpGV-E, is a mixture of at least three genotypes E1, E2 and E3. CpGV-R has a 2.45 kbp deletion when compared to CpGV-M1 (Crook *et al.*, 1985). This is in agreement with Harvey and Volkman (1983). There was no difference found between the isolates CpGV-R and CpGV-M in terms of infectivity (Crook *et al.*, 1985) although Harvey and Volkman (1983) reported a significant difference in their LD_{50} values. Chowdhury (1992) cloned two genotypes R1 and R2 from CpGV-R by *in vivo* cloning and found CpGV-M1 to have a significantly faster speed of kill than CpGV-R1 and CpGV-R2. The sequencing of the CpGV-M1 genome is near completion and most studies have been performed using this genotype.

1.10 The summer fruit tortrix, *Adoxophyes orana*, a lepidopteran pest of apples

Adoxophyes orana is a lepidopteran pest from the family Tortricidae and is more commonly known as the summer fruit tortrix. The caterpillar can develop on many species of forest tree, but prefers apples or secondarily pears and is also a pest of cherries, plums, apricots, red currants, peaches and strawberries.

The summer fruit tortrix has recently become a pest in orchards in the UK. It was first recorded in Kent in 1950 where it had arrived from the continent (Cross, 1994). Summer fruit tortrix is a pest in most European countries and is also widespread in Japan (Cross *et al.*, 1999; Yamada and Oho, 1973). It has now reached orchards all over Kent, Essex and throughout most of East Anglia (Cross, 1994).

Summer fruit tortrix causes considerable damage to fruit, in particular, apples and pears. The larvae feed on the leaves of the trees, which generally does not harm the tree's growth. However, the damage to the fruit can be extensive and costly. The

larvae feed on the epidermis of the fruit leaving lesions which fungi can then invade. These wounds heal poorly leaving corky scars (Cross, 1994). The caterpillars also feed on flower parts especially the receptacles and severely damaged flowers will fall to the ground.

The summer fruit tortrix has two generations a year in the UK and in most European countries. The life cycle for summer fruit tortrix in the UK is described by Cross (1994) but is summarised as follows. The first generation larvae hatch from batches of eggs laid on the undersides of leaves in mid-June. The larvae graze on leaves and fruit. As the larvae grow they characteristically tie a leaf around their bodies with fine webbing which they spin. The caterpillars are often described as leaf rollers due to this. The leaf roll protects the caterpillar from predators and also serves as food. After undergoing five larval instars the caterpillars pupate in late July. The adult moths fly on warm August evenings and lay their eggs directly on the surface of the fruit. The second generation of larvae hatch in August and feed on the fruit and leaves. These caterpillars also roll leaves and will often tie a leaf to the fruit for protection. When the leaves senesce in late September or early October the second or third instar larvae seek overwintering sites. They weave cocoons in the crevices of bark or in the crook of a forking twig or bud axil. In the event of a hot summer some caterpillars may continue to grow and give rise to moths which die without reproducing. The caterpillars that overwinter become active again in the warmer spring months and feed on young sappy bark on the shoots. At or shortly after bud burst, during April and May, the caterpillars move to feed on the blossom trusses. Some larvae hang down on the end of silken threads, and may then be dispersed by the wind, occasionally over long distances.

A number of methods have been employed to try and control summer fruit tortrix. As with most pests, chemical insecticides have been heavily used. These include chemicals such as DDT, pyrethroids and organophosphates (Cross, 1994). Chemical insecticides, although effective, are not species-specific and other beneficial insects may be adversely affected including natural enemies of the caterpillar such as earwigs and lacewings. Therefore, the use of chemicals is not desirable and the withdrawal of chemical insecticides such as organophosphates is already underway. Other methods of control include pheromone traps. These attract the male moths when they fly in the

warm evenings and so can reduce the next generation by preventing mating of the moths. However, this is also not very specific and other species are attracted to the traps.

Another control method is the use of Fenoxycarb (Cross, 1997). Fenoxycarb is a juvenile-hormone analogue. It is more active and easily produced than natural juvenile hormone and it disrupts the fifth instar larvae before they pupate (Lovelidge, 1997). In healthy larvae, juvenile hormone levels remain constant and at a high level until just before pupation when the levels drop dramatically. Fenoxycarb results in the juvenile hormone level remaining high and so pupation cannot occur. This results in larval abnormalities, which are lethal (Cross, 1997). Although Fenoxycarb is much more specific than chemical insecticides it still can affect other insects. One of the insects it affects is bees. Bees can transfer Fenoxycarb to their hives where up to 20% of the brood may die (Lovelidge, 1997). Therefore, Fenoxycarb can only be sprayed when there are no flowers on the apple trees and care must be taken that there are no flowering weeds near the orchard at the time of application (Lovelidge, 1997).

In Switzerland and Germany, a Swiss isolate of a granulovirus of the summer fruit tortrix is registered and used commercially as a biocontrol agent by Andermatt Biocontrol AG. This granulovirus is called *Adoxophyes orana* granulovirus (AoGV). This virus takes much longer to kill larvae than chemical insecticides but appears to have no effect on any other species including natural enemies of the pest. The slow speed of kill can be an advantage as sustained control can be achieved over several generations (Andermatt, 1989).

AoGV has been reported to exhibit two types of cytopathology. An electron microscope study performed by Schmid *et al.*, (1983) indicated that there could be two pathotypes of the Swiss strain of AoGV. The first resulted in a cytopathology typical of normal GV infections whereby the nucleus of fat body cells was infected and the nuclear membrane broke down early in infection. This they named the 'nuclear type'. The second pathotype was named the 'cytoplasmic type'. In this type OBs were seen only in the cytoplasm of fat body cells and the nucleus appeared uninfected and healthy.

There is another species similar to *A. orana* named *Adoxophyes sp.* or the smaller tea tortrix. This caterpillar, as its name suggests, is a pest of tea trees and is particularly prevalent in Japan (Nishi and Nonaka, 1996). There are only slight morphological differences between the two species, and the adults of the species can reproduce but form sterile offspring (Honma, 1970). In Japan an isolate of AoGV isolated from *Adoxophyes sp.* is used as a biocontrol agent of smaller tea tortrix (Nishi and Nonaka, 1996).

An English isolate of AoGV (AoGV-E) was isolated from overwintering caterpillars obtained from two orchards in Kent in 1993. This virus has potential for use as a biocontrol agent in the UK.

1.11 Aims of the thesis

CpGV and AoGV are both produced commercially as biocontrol agents against larvae of the codling moth, *Cydia pomonella* and the summer fruit tortrix moth, *Adoxophyes orana,* respectively. There is currently very little information on GVs compared with NPVs, and only recently has there been an increase in number of laboratories involved in molecular studies on GVs.

- The primary objective of this PhD thesis was to study the relationships between GVs and to investigate factors or genes involved in controlling their host range, tissue tropism and speed of kill. One of the initial aims of this study was to evaluate the relationship between GVs with different tissue tropism and differences in speed of kill.

- This led on to a study of a slow killing granulovirus, AoGV which was found not to be typical of the other slow killing granuloviruses studied to date. Further work on this virus may determine a factor or gene responsible for the differences in speed of kill and tissue tropism.

- From hybridisation studies performed, it was clear that two viruses *Cryptophlebia leucotreta* GV (ClGV) and *Cydia pomonella* GV (CpGV) had a relatively high DNA homology. Therefore, another aim of this project was to determine host range factors of CpGV that would allow ClGV to replicate in *C. pomonella* cells, by producing a recombinant ClGV.

- When recombinant viruses are made, a thorough testing of their host range is required. This led to a project to construct transfer vectors for the production of two granuloviruses containing reporter genes that would allow a comprehensive study on host range to be performed. The resulting viruses would also be useful in studying the course of infection of CpGV in a variety of hosts displaying differing permissivity.

- The final aim of this project was to investigate the role of an extra region of DNA in a CpGV genotype that could have been responsible for an increase in its speed of kill.

CHAPTER 2

Materials and Methods

2.1 Chemicals

Chemicals were supplied by Sigma Aldrich Chemical Company and BDH Laboratory Supplies. Restriction endonucleases and DNA modifying enzymes were obtained from Roche Diagnostics Ltd and GIBCOBRL®. Amersham Pharmacia Biotech Inc. supplied the nucleotide (^{32}P) dCTP. Nylon hybridisation membranes were PALL BIODYNE® A supplied by GIBCOBRL®. Disposable sterile plasticware was obtained from Sterilin Ltd. All water used was from the Milli-Q® water purification system or Elga Maxima Ultrapure water.

2.2 Restriction enzyme analysis of DNA

2.2.1 Restriction digestion

DNA was digested using restriction enzymes and reaction buffers, according to the manufacturers recommendations.

2.2.2 Agarose gel electrophoresis

For virus DNA 0.7% (w/v) agarose gels were prepared in 1 x Tris-acetate/EDTA buffer (TAE) diluted from a 10 × stock. For gels used in a Bio-Rad mini-sub™ DNA cell, 30 ml melted agarose was required, 60 ml was required for a Bio-Rad wide mini-sub™ DNA cell and 70 ml for gels used in BRL model H5 electrophoresis tank. The gels were then run at constant voltage of 20 V for 16 h (overnight). For plasmid DNA and PCR products 1-1.5% (w/v) agarose gels were prepared in 1 x TAE and run for 1-4 h at 70 V.

2.2.3 Staining and photography of the gel

Gels were stained by soaking in 0.5 µg/ml ethidium bromide for 20 min. The DNA was visualised on a short wave UV trans-illuminator at 312 nm (Appligene) and pictures were taken using a COHU High Performance CCD video camera or a Polaroid camera, loaded with Polaroid 665 positive/negative film.

2.2.4 Determination of the sizes of restriction fragments

DNA standard ladders were run on all gels to estimate the fragment sizes. The standards used were all supplied by GIBCOBRL®. Namely '1 Kb DNA ladder', 'λ *Hin*dIII' and 'High molecular weight DNA marker'. These were diluted to 600 ng/10 µl in TE. These standard DNA fragment sizes and their mobilities were used to predict the size of unknown fragments (Schaffer and Sederoff, 1981).

2.2.5 Extraction of DNA with phenol:chloroform

Extraction with phenol:chloroform was performed as described by Sambrook *et al.* (1989).

Briefly, an equal volume of phenol:chloroform was added to the DNA sample in an Eppendorf tube. The contents were mixed until an emulsion formed. The mixture was centrifuged in a microfuge at 13,000 rpm for 1 min. The aqueous phase was transferred to a fresh tube. An equal volume of chloroform:isoamylalcohol (24:1) was added and mixed and centrifuged as above. The aqueous phase was then transferred to a fresh tube.

2.2.6 Precipitation of DNA with ethanol

Ethanol precipitation was performed as described by Sambrook *et al.* (1989). Briefly, sodium acetate pH 5.2 was added to the DNA solution to give a final concentration of 0.3 M. Then two volumes of ice-cold ethanol was added to the DNA solution and mixed well. The DNA was kept on ice or at –20°C for approximately 30 min. The mixture was then centrifuged in a microfuge at 13,000 rpm for 10 min. The supernatent was carefully removed and 500 µl 70% ethanol added to the tube. The tube was centrifuged as above for two min. The 70% ethanol was removed and the lid of the tube left open in a 37°C incubator for 5 min to allow all of the fluid to evaporate. The DNA pellet was then redissolved in the desired volume of 1 x TE pH 8.0. For very small amounts of DNA 20 µg glycogen was added to the DNA prior to ethanol precipitation as a coprecipitate.

2.2.7 Recovery of DNA fragments from agarose gels

This was performed using either QIAquick Gel Extraction Kit from Qiagen or Geneclean®II Kit from BIO 101 Inc. according to manufacturers recommendations. Alternatively, an electroelution method was used described by Sambrook *et al.* (1989). After electroelution the DNA was extracted with phenol:chloroform and precipitated with ethanol. The DNA pellet was resuspended in 1 × TE (pH 8.0).

2.3 Cloning DNA fragments

2.3.1 Quantitative analysis of DNA

A Pharmacia GeneQuant RNA/DNA calculator was used to determine the concentration and purity of DNA in a sample by reading the wavelength of the sample at 260 nm and 280 nm. An OD at 260 nm of 1 = 50 µg/ml for double stranded DNA. Pure DNA had a (OD_{260}/OD_{280}) ratio of 1.8. A value lower indicated the presence of proteins or phenol. A higher value indicated the presence of RNA.

2.3.2 Culturing of *E. coli*

Luria Bertani media (LB) was used for the culturing of *E. coli*, Sambrook *et al.* (1989). Cultures of *E. coli* were cultured at 37°C, either in liquid cultures in a shaking incubator at 200 rpm or on LB agar plates. The appropriate antibiotics were added to the media to ensure plasmid maintenance.

2.3.3 Preparation of plasmid DNA

2.3.3.1 Small scale (2 ml culture)

DNA was prepared from plasmid DNA by alkaline lysis as described by Sambrook *et al.* (1989), except RNAase A was added to solution 1, to give a final concentration of 400 µg/ml. The DNA was finally redissolved in 50 µl of sterile TE (pH 8.0), without RNAase A.

2.3.3.2 Medium (25 ml culture) and large scale (100 ml culture)

DNA was prepared using QIAGEN-tip 100 and 500 respectively according to the manufacturers recommendations.

2.3.3.3 Preparation of endotoxin-free DNA

Endotoxin-free DNA was prepared using Qiagen-tip 500 in the EndoFree™ Plasmid Maxi Kit according to the manufacturers recommendations.

2.3.3.4 Preparation of cosmid DNA (500 ml culture)

DNA was prepared from cosmids by alkaline lysis as described by Sambrook *et al.* (1989). The resulting DNA was redissolved in 3 ml of sterile TE (pH 8.0). The DNA was then purified by equilibrium centrifugation in caesium chloride (CsCl)-density gradients (section 2.3.4).

2.3.4 CsCl-density gradients

CsCl-density gradients were prepared according to Sambrook *et al.* (1989). For purification of viral DNA, 1.1 g of solid CsCl was added for every 1 ml of DNA solution. The gradients were assembled in Beckman Quick-Seal tubes and centrifuged in a Beckman vertical 80Ti rotor. Ethidium bromide was extracted from the DNA with water-saturated 1-butanol as described in Sambrook *et al.* (1989).

2.3.5 End-filling cohesive ends

Approximately 5 µg DNA was digested in a volume of 20 µl with the appropriate restriction enzyme. The DNA was heated for 15 min at 65°C to inactivate heat sensitive restriction enzymes or phenol:chloroform extracted to inactivate heat resistant restriction enzymes. A volume of 2 µl of each dNTP (2mM) was added to the DNA. Ten units of Klenow fragment were added and the reactions incubated at 20°C for 25 min. The enzyme was then inactivated by adding 1 µl 0.5M EDTA. The DNA was either phenol:chloroform extracted and ethanol precipitated, or separated on an agarose gel and the desired DNA fragment excised, purified and redissolved in water.

2.3.6 Production of blunt ends using mung bean nuclease

Approximately 10 µg DNA was digested with the appropriate restriction enzymes. The DNA was then phenol:chloroform extracted and ethanol precipitated or excised from a gel and purified. The DNA was then redissolved in 89 µl TE pH 8.0. A volume of 10 µl, 10 x mung bean nuclease buffer (300mM NaAc pH 4.6, 500mM

NaCl, 10mM $ZnCl_2$) was then added. After which 1 µl mung bean nuclease (10 units/µl) freshly diluted in 1 x mung bean nuclease buffer was added. The reaction was incubated at 37°C for 10 min. A volume of 4 µl 10% SDS was then added to inactivate the enzyme. The DNA was phenol:chloroform extracted, ethanol precipitated and redissolved in water.

2.3.7 Ligation of phosphorylated linkers to blunt ended DNA

The following were added to an Eppendorf tube:

500 ng DNA

Phosphorylated linkers 0.5-1 µg

10 mM ATP

Water to 17 µl

2 µl 10 x ligase buffer

1 µl T4 DNA Ligase (10 U)

Two reactions were set up using either a 1:500 or 1:1000 molar ratio of DNA to linker. The DNA was left to ligate overnight (16 h) at 25°C. The reaction was then heated at 65°C for 15 min to inactivate the enzyme. The reaction was chilled on ice and then 70 µl water and 10 µl 10 x restriction enzyme buffer was added. Thirty units of restriction enzyme were added and the reaction was incubated for 4 hours at 37°C. Ten more units of enzyme were added and the reaction was incubated for a further hour. The reaction was then extracted with phenol:chloroform and precipitated with ethanol with the addition of 1 µl glycogen.

2.3.8 Ligation of DNA into a plasmid vector

Ligation reactions were performed using GIBCOBRL® T4 DNA ligase according to the manufacturers recommendations. Approximately 50 ng dephosphorylated vector DNA (section 2.3.9) was used in the ligation reactions and a molar ratio of 3:1 insert:vector was used except for shotgun cloning of entire granulovirus genomes. In these cases, 500 ng of digested viral DNA and 50 ng dephosphorylated vector DNA was used, which had been found to be optimal.

2.3.9 Dephosphorylation of DNA

DNA was dephosphorylated using shrimp alkaline phosphatase (Roche Diagnostics Ltd) according to manufacturers recommendations. Shrimp alkaline phosphatase was used because it could be heat inactivated.

2.3.10 Transformation of *E. coli* strains

An overnight culture of *E. coli* was cultured in LB broth. For every electroporation required 1ml culture was added to 4 ml LB broth. This was cultured at 37°C for 3 h so that the cells were in exponential phase. The cells were then centrifuged in an MSE Mistral 1000 centrifuge at 720 *g* for 5 minutes. They were washed and pelleted three times in refrigerated water and resuspended in 50 µl water per electroporation required. A volume of 40 µl was then added to chilled cuvettes (Bio-Rad, *E.coli* pulser cuvettes 0.2 cm electrode gap). The electroporator used was Bio-Rad gene pulser™ with pulse controller. The settings used were as follows:

25 UF

200 Ohms

2.5 kV

Once electroporated, 1ml LB broth was added to each cuvette and they were then incubated at 37°C for 1 h. A volume of 100 µl of cells were then plated onto LB agar plates containing the required antibiotics and substrates.

2.4 Sequencing

Double stranded DNA sequencing was performed utilising the dideoxynucleotide chain termination method, Sanger *et al.* (1977).

Sequencing reactions using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit were set up according to Perkin-Elmer Applied Biosystems. For PCR products approximately 90 ng DNA was used. For plasmid DNA approximately 500 ng Qiagen prepared DNA was used. The sequencing reactions were carried out using the Gene Amp PCR Systems 9600 and visualised on the ABIPrism® 377 DNA Sequencer, using Filter Set E.

2.5 Nucleic acid hybridisation

2.5.1 Southern hybridisation (capillary transfer)

After gel electrophoresis, DNA fragments were capillary blotted onto nylon membrane using a procedure adapted from Sambrook *et al.* (1989).

The gel was soaked in depurination solution (0.25M HCl) for 10 min with gentle shaking, then washed in distilled water. It was then soaked in denaturation solution (1.5M NaCl, 0.5M NaOH) for 20-30 min. Three pieces of 3MM Whatman® filter paper, 1 cm bigger than the gel on all sides was soaked in 20 x SSC and placed on a piece of clingfilm. The gel was then placed on the filter paper and the nylon membrane, cut to the size of the gel, placed on top of the gel. The edges of the clingfilm were folded over the nylon membrane overlapping by about 0.5 cm. Three pieces of 3MM Whatman® paper were cut to the same size of the gel and placed on top of the nylon membrane. Approximately 5 cm thickness of paper towels were placed on the top of the apparatus and a weight was placed on the paper towels. The apparatus was then left overnight. The next day the nylon membrane was air-dried and the DNA fixed to the membrane by crosslinking on a UV transilluminator (Appligene) at 0.35 J/cm^2 of UV light.

2.5.2 Southern hybridisation (vacuum transfer)

Nylon membrane was cut 1 mm short of the edges of the window in a plastic mask. The membrane was soaked in 20 × SSC for 30 min. The blotter was set up and the treated membrane positioned in the window. The gel was then placed onto the membrane, overlapping the window by about 1 mm on all sides. The pump was switched on and the vacuum adjusted to 40 cm H_20. Approximately 15 ml 0.25M HCl was added to cover the gel. After 4 min it was drawn off and replaced by 1.5M NaCl, 0.5M NaOH for 3 min followed by 1M Tris-HCl pH 8.0, 1.5M NaCl for 3 min. Finally water was added to cover the gel and left for 30 min. The water was topped up as necessary, then removed. The gel was peeled off with the vacuum still on and viewed on a UV transilluminator to confirm that the transfer had been successful. The pump was switched off and the membrane removed, air dried and fixed by crosslinking at 0.35 J/cm^2 of UV light.

2.5.3 Dot blotting

Dot blotting was performed using Bio-Rad dot blot apparatus. A piece of nylon membrane the size of the 96 well plate was cut out and soaked in 1M NH_4 acetate, 0.02M NaOH. The membrane was then placed in the blotter. The medium from insect cells was removed and the cells lysed by the addition of 200 µl/well of 0.5M NaOH. A volume of 50 µl of 5M NH_4 acetate was then added to each well and 100 µl of the lysate added to the corresponding well of the blotter. The vacuum was turned to a suction pressure of 100 cm H_2O. When the lysate had been drawn through the nylon membrane, the vacuum was released and the blot placed in a Whatman® 3MM filter paper envelope to air dry. The blot was fixed by crosslinking at 0.35 J/cm^2 of UV light.

2.5.4 Hybridisation of radiolabelled DNA probes to DNA on nylon membranes

Hybridisation reactions were performed in polypropylene boxes in a shaking Hybaid MAXI 14 hybridisation oven. High stringency hybridisations for homologous probes were carried out at 65°C and low stringency for heterologous probes at 55°C. The DNA probes were labelled with ^{32}P dCTP by random priming using the HYPER-Prime™ Random Primed DNA Labelling Kit (Bioline) according to the manufacturers recommendations. The pre-hybridisation and hybridisation solutions were those described in Sambrook *et al.* (1989) except 5 x SSC was used instead of 6 x SSC. (5 x SSC, 5 x Denhardt's reagent, 0.5% SDS and 100 µg/ml denatured herring sperm DNA).

Post hybridisation washes were performed with 2 x SSC, 0.1 % SDS for 30 min at room temperature, 1 x SSC, 0.1 % SDS for 30 min at hybridisation temperature and 0.1 x SSC, 0.1 % SDS for 30 min at hybridisation temperature.

2.5.5 Hybridisation of DIG labelled DNA probes to DNA on nylon membranes

DNA probes were labelled using the DIG-High Prime DNA labelling and Detection Starter kit II (Roche Diagnostics Ltd) and hybridisations were performed according to the manufacturers recommendations. Hybridisations were carried out in hybritubes (Hybaid) in a shaking water bath. Post-hybridisation washes were performed as in section 2.5.4 in the hybritubes and the detection procedure was carried out in clean plastic trays.

2.6 Infection of larvae with virus

2.6.1 Insect rearing on semi-synthetic diet

Clean laboratory stocks of *Cydia pomonella*, *Adoxophyes orana* and *Cryptophlebia leucotreta* were maintained in an insect rearing unit. Eggs were routinely surface sterilised in vapour from 5% (v/v) formaldehyde. Stock *C. pomonella* and *A. orana* were reared individually in 'polypots'. *C. leucotreta* were mass reared in plastic lunch boxes. The constituents of the semi-synthetic diet, used for rearing larvae, are listed in Appendix 1. The diet for rearing was supplemented with formaldehyde to reduce microbial contamination of the diet. The diet prepared for larvae used for amplification of virus or haemolymph production was supplemented with juvenile hormone to prevent pupation and maximise virus yield. 'Water diet' containing extra water was used for neonate bioassays in 96 well plates.

2.6.2 Determination of the volume of liquid ingested by neonates

The method used to determine the volume ingested by neonate larvae was based on fluorescence spectroscopy developed by van Beek and Hughes (1986). Phenol red (0.3%) was substituted for the blue food colouring, as it was more easily observed in the gut.

2.6.3 Determination of virus mortality following droplet feeding inoculation

The method used to feed neonate larvae was developed by Hughes and co-workers (Hughes and Wood, 1981; Hughes *et al.*, 1986).

A square of parafilm was used as a hydrophobic surface. It was placed in a petri dish on to which a ring of droplets each consisting of a 50:50 mixture of virus solution of known concentration: food dye (0.3% Phenol red) was prepared. The 0-6 hour old neonate larvae were placed into the centre of the ring and allowed to drink to satiation. The larvae that had ingested virus were transferred to a semi-synthetic diet and monitored either daily or 6 hourly depending on the bioassay. *C. pomonella* were monitored at 6 hourly intervals as these would normally die within a few days. *A. orana* were monitored daily as these would take several weeks to die. For bioassays of *C. pomonella* larvae, a blue dye (FD+C blue) was incorporated into the diet to aid visualisation of the larvae. The constituents of the diet were as follows:

30 g semi-synthetic water diet, 40 mg FD + C blue food colouring, 10 ml water

This mixture was then liquefied in a pressure cooker for 15 min and syringed into individual wells of a round bottomed 96 well plate. The diet was allowed to set and neonates were then transferred to individual wells. The plate was covered with clingfilm and then covered with glass slides held on by elastic bands to prevent the larvae from escaping from one well to another. For daily bioassays of *A. orana*, larvae were transferred to individual wells of a 25 well repli-plate with individual cubes of semi-synthetic diet containing formaldehyde.

2.6.4 Mortality determination in fourth and fifth instar larvae

Larvae used for bioassays were reared from eggs laid over a 2-3 day period. The instar of the larvae was determined based on head capsule measurement. Larvae that had reached the desired instar 24 hours prior to the start of the bioassay were discarded. This ensured that larvae used for the bioassay had reached the required instar in the previous 24 hours. Small cylinders of semi-synthetic diet were cut with a No. 1 cork borer and thin slices placed onto a drop of solidified 1% technical agar, in individual wells of a 25 well repli-plate. Virus used for bioassays had been freeze-thawed only once and sonicated prior to use. A volume of 1 µl virus solution at a pre-determined concentration was dispensed onto each slice of diet. The larvae were then placed individually into the wells and left for 24 hours. Larvae that had not consumed the entire diet plug after 24 hours were excluded from the experiment. The larvae were then placed into a fresh 25 well repli-plate containing a cube semi-synthetic diet containing formaldehyde. This ensured that the larvae did not re-ingest any frass, which could still contain infectious virus. The larvae were then monitored until death or emergence as healthy adults.

2.6.5 Cloning virus by limiting dilution *in vivo*

This was carried out as described in Smith and Crook (1988). The virus was diluted to concentrations known to kill about 5-20%. CpGV was cloned using fifth instar larvae. AoGV was cloned using neonate larvae as these would not die until in fifth instar. Single insect OB purifications were performed on the larvae (section 2.7.1.2) and DNA extractions (section 2.7.4) to determine the restriction endonuclease profile

of the virus. The process was repeated three times after the virus appeared homogenous.

2.7 Viruses

2.7.1 Occlusion body (OB) purifications

All apparatus used during OB purification were decontaminated by soaking in 5% formaldehyde for 1 h, rinsing thoroughly in distilled water and autoclaving. All work involving OBs was carried out in a room dedicated to virus work which was fitted with a UV lamp and UV treated after use.

2.7.1.1 Large scale purification

Larvae were macerated with a pestle and mortar in approximately 30 ml 0.1% SDS. A crystal of phenylthiocarbamide (PTC) was added to prevent melanisation. The preparation was then filtered through a layer of miracloth (CALBIOCHEM®). The filtrate was added to a Sorvall 50 ml centrifuge tube and filled with 0.1% SDS. The filtrate was centrifuged at 9200 *g* (10,000 rpm) in a fixed angle SS34 rotor in a Sorvall RC-5B superspeed centrifuge (Sorvall fixed angle) for 30 min. The pellet was resuspended in 0.1% SDS and loaded onto a continuous 30-80% glycerol in 0.1% SDS (v/v) gradient made up in 70 ml tubes. The gradient was centrifuged in an MSE Prepspin 50 Ultracentrifuge using a swing-out rotor (MSE swing-out) at 28,000 *g* (15,000 rpm) for 12 min. The OB band was recovered from the gradient, diluted in 0.1% SDS and pelleted for 30 min at 20,800 *g* (15,000 rpm) using the Sorvall fixed angle rotor. The pellet was resuspended in 0.1% SDS and loaded onto a continuous 45-60% sucrose gradient in 0.1% SDS (w/w). The gradient was centrifuged for 1 h at 50,000 *g* (20,000 rpm) using the MSE swing-out rotor. The OB band was recovered, diluted in water and centrifuged at 20,800 *g* (15,000 rpm) using the Sorvall fixed angle rotor for 30 min. The virus was then washed twice more in water. The pellet was resuspended in water and stored at -20°C.

2.7.1.2 Single insect OB purification

Single insects were macerated using a tissue grinder in 0.5 ml 0.1% SDS in a 1.5 ml Eppendorf tube. The tubes were centrifuged for 10 s at 6500 rpm in a MSE bench microcentrifuge to pellet the larval debris and the supernatant was transferred to a

fresh tube. The larval debris was resuspended in 0.3 ml 0.1% SDS to extract any remaining virus and centrifuged as above. The supernatants were combined and then loaded onto a continuous 30-80% glycerol gradient in 0.1% SDS (v/v) made up in 25 ml tubes. The gradient was centrifuged in an MSE Prepspin 50 Ultracentrifuge swing-out rotor at 23,000 *g* (15,000 rpm) for 12 min. The OB band was recovered from the gradient, diluted in 0.1% SDS and pelleted for 30 min at 20,800 *g* (15,000 rpm) using a fixed angle SS34 rotor in a Sorvall RC-5B superspeed centrifuge. The pellet was resuspended in 0.7 ml 0.1% SDS and loaded onto a continuous 45-60% sucrose gradient in 0.1% SDS (w/w) made up in 25 ml tubes. The gradient was centrifuged for 1 h at 42,000 *g* (20,000 rpm) using the MSE swing-out rotor. The OB band was recovered, diluted in water and centrifuged at 20,800 *g* (15,000 rpm) in the Sorvall fixed angle rotor for 30 min. The virus was then washed twice more in water. The pellet was resuspended in 100 µl of water and stored at –20°C.

2.7.1.3 Crude single insect OB purification

Single insects were macerated using a tissue grinder in 0.5 ml 0.1% SDS in a 1.5 ml Eppendorf. The tubes were centrifuged for 10 s at 6500 rpm in a MSE bench microcentrifuge to pellet the larval debris. The supernatant was transferred to a fresh tube and the larval debris resuspended in 0.3 ml 0.1% SDS to extract any remaining virus and centrifuged as above. The supernatants were combined and centrifuged for 2 min and the virus pellet was resuspended in 100 µl water.

2.7.2 Estimation of virus yield using a spectrophotometer

A dilution of the virus was performed (1:100-1:500) in water so that the suspension appeared slightly cloudy. The absorbance was read at 350 nm and at 260 nm. The following method, determined by Dr. N. E. Crook, was performed to estimate the protein concentration.

(OD 350 nm / 13) × dilution factor = mg/ml

(OD 260 nm / 31) × dilution factor = mg/ml

The average of the two was taken. To estimate the number of OBs;

1 mg/ml = 3.83×10^{10} OBs/ml.

2.7.3 Counting OBs using a counting chamber

For granuloviruses a 0.01 mm^3 Helber 0.01 mm depth counting chamber was used. The virus was diluted to approximately 0.01mg/ml in water. A volume of 2 µl was placed onto the slide and covered with a toughened coverslip and pressed until Newton's rings appeared. The OBs were observed using a light microscope under dark field illumination. The OBs were counted in four large diagonal squares (64 small squares) and if the count was less than 100 the other diagonal was also counted. This was repeated for another 2 µl sample. Another virus dilution was made and two further virus counts were carried out.

2.7.4 Extraction of viral DNA

This method is outlined for 500 µl purified capsules and was adjusted according to the volume of virus used.

The concentration of OBs was adjusted to approximately 10 mg in 500 µl. A volume of 25 µl 1M Na_2CO_3 was added and the suspension incubated for 30 min at 37°C. The pH of the clarified suspension was adjusted to pH 8.0 with the addition of approximately 20 µl 1M HCl. A volume of RNAse A containing 25 µg was added and incubated for 10 min at 37°C. Then 60µl 10% SDS and 125 µg Proteinase K were added and the mixture incubated for 1 h at 37°C. The mixture was then phenol:chloroform extracted. The aqueous phase was transferred to a dialysis bag or chamber and dialysed against 1 litre 1 × TE pH 8.0 for at least 24 h with three changes of buffer. The dialysed DNA was then transferred to a fresh microcentrifuge tube and stored at -20°C.

2.8 Cell culture

2.8.1 Cells

Primary *Cydia pomonella* cells were derived from embryos at Horticulture Research International (Winstanley and Crook, 1993). The cell lines used were CpDW14, which was maintained at 19°C and CpDW14R, which had been retinoic acid treated and were maintained at 27°C. The CpDW14R cells were faster growing but the CpDW14 cells were believed to produce more budded virus.

2.8.2 Media

The *C. pomonella* cells were maintained in IZDO4 medium (Winstanley and Crook, 1993), modified from IZD03 medium (Miltenburger *et al.,* 1984), developed at the Institute of Zoology, Darmstadt, for their *C. pomonella* 3300 cell line. Media compositions are shown in Appendix 2. After the medium had been made it was filter sterilised through a 0.2 µm filter and checked for sterility by transferring 2 ml to a sterile bijou and incubating in a shaking incubator at 37°C overnight. Foetal bovine serum was heat treated at 50°C for 30 min before adding to IZDO4 at 10%. All media were kept at 4°C. When antibiotics were required, 50 U/ml penicillin and 50 µg/ml streptomycin were added to the medium.

2.8.3 Subculturing cells

The following is described for 25 cm^2 flasks. For 75 cm^2 flasks all volumes were tripled.

Cells were passaged when confluent. The medium was removed and 2 ml fresh medium added. The cells were detached from the bottom of the flask with a cell scraper. They were then aliquoted and dispensed into the required number of flasks (usually a 1:4 split). Fresh medium was then added to the cells to a volume of 5 ml. The flasks were then swirled gently to mix the cells and medium and placed at the required temperature.

2.8.4 Counting cells and determination of cell viability

Cells were counted using a Neubauer haemocytometer. The viability of the cells was determined by the use of trypan blue exclusion. The cell suspension was diluted 1:1 in 0.2% Trypan Blue. Non-viable cells took up the blue coloration.

2.8.5 Production of infectious haemolymph for *in vitro* infection

Larvae were infected with an approximate LD_{95} dose. Haemolymph was collected at exactly 3 d p.i. Larvae were anaesthetised by exposing to diethyl ether vapour for 2-3 min. The proleg was pricked with a syringe needle and haemolymph gently squeezed out and drained onto a sterile petri dish. It was then transferred using a micropipette

into a sterile bijou bottle containing a small crystal of PTC and 1 ml cell culture medium. A final dilution of 1:10 of the haemolymph to cell culture medium was made. The haemolymph was filtered through a 0.45 μM filter (PALL Gelman Sciences) and stored at –70°C, or at 4°C if being used within a few days.

2.8.6 Infection of cells with infectious haemolymph

The following is described for 25 cm^2 flasks, for 75 cm^2 flasks all volumes were tripled. Cells were seeded at 2 $\times 10^6$ viable cells. The cells were left to attach overnight for CpDW14R cells and 4 days for CpDW14 cells at 27°C. The medium was removed and replaced with 1 ml infectious haemolymph. The flasks were rocked gently for 4 h at 25°C in the dark. The haemolymph was removed and fresh complete medium, containing antibiotics added. The cells were then incubated at 27°C. After 9 days, 4 ml medium was removed, aliquoted and stored at 4°C or –70°C and 4 ml fresh medium was added to the cells. At 12 d p.i. the medium was removed again and stored as above. The medium removed was referred to as passage one virus (P1).

2.8.7 Infection of cells with passage 1 (P1) virus

Infection of cells with P1 virus was carried out in the same way as infection with haemolymph. The medium was removed at 6-7 d p.i. and again at 10-12 d p.i. and was referred to as passage 2 virus (P2).

2.8.8 Transfection of cells with DNA.

Plasmid or cosmid DNA used for transfection had either been purified using a Qiagen EndoFree™ Plasmid Maxi Kit or by CsCl-density gradient equilibration. Freshly prepared viral DNA purified from OBs using the method previously described (section 2.7.4) was used directly or was further purified by CsCl-density gradient equilibration (section 2.3.4). Transfection reagents used were DOTAP Liposomal Transfection Reagent (Roche Diagnostics Ltd) and Insectin-Plus™ Insect Cell-Specific Lipids (Invitrogen). The transfection reagents were used according to the manufacturers recommendations. The transfection medium was made up containing 1 ml serum free cell culture medium, approximately 5 μg DNA and the recommended volume of transfection reagent. This mixture was then precomplexed for 15 min. Cells were transfected at a density of 2 $\times$ 10^6 viable cells per 25 cm^2 flask, using the 1

ml transfection medium for four hours at 25°C with gentle rocking. The transfection medium was removed and replaced with 5 ml complete medium containing antibiotics. When transfections and infections were performed, the cells were first infected with P1 virus for 4 h then immediately transfected for 4 h with DNA.

2.8.9 Cloning virus by limiting dilution

A dilution series of passaged virus was prepared in 96 well plates and then each well was seeded with 2×10^4 cells. Duplicate columns were used for each concentration. The dilution series was usually from 1/100-1/3200. The dilutions were altered when required. The plates were incubated at 27°C. A volume of 50 µl medium was removed from each well 6-7 d p.i. and stored at 4°C or –70°C and 100 µl complete medium was added to replace the 50 µl removed and to allow for any evaporation. At 10-12 d p.i. the medium was removed again and stored as above. Half of the cells were stored at –20°C for feeding to larvae and the other half were used for dot blotting.

2.9 Ecdysteroid UDP-glucosyl transferase (EGT) enzyme assay

Baculovirus infected cells were collected from a 25 cm^2 flask (2×10^6 cells) and centrifuged in an MSE mistral 1000 at 920 *g* (4500 rpm) for 5 min to pellet the cells. They were resuspended in 1 ml 1 × TMM (10 mM Tris-malate pH 7.4, 10 mM $MgCl_2$). The cells were then lysed with a hand held glass homogeniser on ice. When haemolymph was used it was collected from larvae and transferred immediately into an Eppendorf tube on ice containing a crystal of PTC. A volume of 50 µl resuspended cell homogenate or 20 µl haemolymph was used for each assay made up to a final volume of 100 µl. The following components were added to Eppendorf tubes:

10 µl 10 × TMM, 10 µl 10mM UDP-Glucose, 10 µl 10mM UDP-Galactose, 50 µl cell extract or 20 µl haemolymph, 2.5 µl ^{3}H-ecdysone, water to 100 µl.

The contents of the tubes were mixed and centrifuged briefly, then incubated at 37°C for 1 h. The reaction was stopped by the addition of 200 µl of cold 95% ethanol. The samples were evaporated to dryness in a speed vacuum centrifuge (Uniscience,

Univap) with the Eppendorf lids open and a temperature of 40-45°C. The samples took 2-2.5 h to dry. The dry samples were resuspended in 50 µl 60% ethanol and left at 4°C overnight. A total volume of 21 µl of the samples (in 3 µl aliquots) were spotted onto thin layer chromatography (TLC) plates coated with a silica gel, 1.5 cm apart and 4 cm from the bottom of the plate. The TLC plate was placed in a chromatography tank containing the following solvent mixture to a level 5 mm under the dots.

n-butanol : acetone : glacial acetic acid : 30% ammonia : water (70 : 50 : 18 : 1.5 : 60)

The TLC plate was left for approximately 3.5 h until the buffer had drawn up to about 1 cm from the top, after which it was removed from the tank and air dried. The plate was then exposed to ^{3}H-sensitive phosphorimager screen overnight. The resulting image was viewed on a phosphorimager.

2.10 PCR

PCR reactions were performed using Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech Inc.) according to the manufacturers recommendations and DNA was amplified in a Hybaid Omnigene PCR machine using the amplification conditions described below;

Temp	Time		Cycles
95°C	2 min		1
95°C	45 s	}	
60°C	45 s	}	35
72°C	45 s	}	

2.11 Embedding tissues for electron microscopy

Larvae were anaesthetised by exposure to ether vapours for 2-3 min. The larvae were then dissected under fixative of 2.5% glutaraldehyde in 0.2M cacodylate buffer pH 7.0. The tissues for embedding were cut into small pieces. Fixation was performed at 4°C for 1 h. The primary fixative was rinsed off with three washes (10 min each) of buffer. A secondary fixation of 0.5% osmium tetraoxide for 30 min was then carried

out followed by four buffer rinses of 10 min each. The samples were then dehydrated in an ethanol series at room temperature of 30 min in each (30, 50, 70, 80, 90% ethanol) followed by three changes of 100% ethanol. For the final change the ethanol was dried over a molecular sieve. The samples were infiltrated with a series of increasing concentrations of Spurr's resin (Spurr, 1969) in ethanol (25, 50, 75, 100%) over a period of 7 days. The samples were embedded in fresh 100% Spurr's resin and polymerised at 70°C for 9 h. Blocks were trimmed and 1 µm 'survey' sections for light microscopy were taken in order to select regions of interest for ultrastructural studies. For electron microscopy ultrathin sectioning was performed using a Reichert Ultracut E ultramicrotome. Sections approximately 70 nm thick were collected on copper grids and stained with lead citrate (Reynolds, 1963) and uranyl acetate. The sections were observed in a JEOL 100CX transmission electron microscope and photographed onto Kodak 4489 film.

2.12 DNA analysis

Double stranded DNA sequences were assembled by using the SeqMan II sequence analysis package (Lasergene software; DNAstar, Inc., Madison, Wis.). The coding regions were predicted using the package GeneQuest™ II (DNAstar) by locating translation start and stop codons of ORFs over 50 amino acids. Database searches using the BLASTP protocol were used to identify similar ORFs. Percent pairwise identities were calculated using the GAP program of Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc. (Devereux *et al.*, 1984) on its default settings. The MegAlign package (DNAstar) was used to prepare multiple sequence alignment files (MSF) via the Clustal V algorithm (Higgins and Sharp, 1988). MSF alignments were analysed by using PaupSearch and PaupDisplay of Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc. Unrooted trees were generated using maximum parsimony and the 'branch-and-bound' search program with 1000 bootstrap replicates. Parsimony trees were prepared by using TreeView (Page, 1996).

CHAPTER 3

Genome similarities among granuloviruses

3.1 Introduction

Southern blot hybridisation studies were carried out to gain information on the genomic relationship between a variety of fast and slow GVs (Table 3.1.) (sections 2.5.1 and 2.5.2). The genotypes of the granuloviruses used and the source of the viruses are shown in Table 3.2.

A set of CpGV (fast GV) overlapping cosmids, together covering the entire CpGV genome, were used as probes against digests of the slow GVs AoGV, TnGV and XcGV, and digests of the fast GVs ClGV, PrGV, LoGV and PxGV. Hybridisation studies were also carried out between fragments of TnGV DNA, covering 96.9 % of the genome and restriction digests of XcGV, ClGV and AoGV. These were performed to determine the degree of similarity between TnGV and another slow GV and secondly to compare TnGV to a fast GV. The degree of hybridisation between AoGV and fast or slow GVs would indicate the degree of similarity between these viruses.

These hybridisation studies were to determine whether there were blocks of genes in GVs that were collinear. If there were this would indicate that the structural organisation of the viral genomes had been conserved throughout evolution. It has already been found that the granulin-coding region as well as two other regions showed collinear arrangement between ClGV and CpGV (Jehle *et al.*, 1992). The genomes of PxGV and XcGV, which have both been completely sequenced, have also been reported to show relatively similar gene arrangements (Hashimoto and Hayakawa, 1999). These studies show how closely related fast and slow GVs are to themselves and each other and identify where variation may occur.

Table 3.1

The abbreviations of viruses used in this study are given below. Also the host in which the viruses were isolated, the family of the host, whether the virus is fast or slow to kill, the tissues that are infected and the genome sizes of the viruses.

Host species	Family	Abbreviation of GV	Fast or slow killing	Tissues infected	Genome size of GV (kbp)
Cydia pomonella	Tortricidae	CpGV	Fast	All major tissues	125.6
Cryptophlebia leucotreta	Tortricidae	ClGV	Fast	All major tissues	112.4
Pieris (Artogeia) rapae	Pieridae	PrGV	Fast	All major tissues	106.5
Plutella xylostella	Yponomeutidae	PxGV	Fast	All major tissues	101.0
Laconobia oleracea	Noctuidae	LoGV	Fast	All major tissues	133.5
Adoxophyes orana	Tortricidae	AoGV	Slow	Fat body	100.9
Trichoplusia ni	Noctuidae	TnGV	Slow	Fat body	175.6
Xestia c-nigrum	Noctuidae	XcGV	Slow	Fat body	178.7

Table 3.2

Particular genotypes used in these studies and the source of virus

GV genotype	Source of virus
CpGV-M1	Horticulture Research International, Wellesbourne, UK (HRI)
ClGV-CV3	Dr. J. Jehle, SLFA Neustadt, GERMANY
PrGV (ArGV-5)	HRI
PxGV-J	Dr. Y. Hashimoto, Kyoto Institute of Technology, JAPAN
LoGV-S	HRI
AoGV-E	HRI
TnGV	Dr. Y. Hashimoto, Kyoto Institute of Technology, JAPAN
XcGV-α4	Dr. C. Goto, National Agriculture Research Center, JAPAN

3.2 Results

The CpGV cosmid library had been constructed by ligating CpGV DNA, partially digested with *Sal*I to pVK102 cosmid DNA digested with *Sal*I (Crook *et al.*, 1997). Cosmids used as probes in the hybridisation experiments were M73, M64, M65, M17, M69 and M2, Figure 3.1A. The set of overlapping cosmids covered the entire CpGV genome. Fragments of TnGV DNA covering 96.9 % of the TnGV genome were also used as probes, Figure 3.1B. These were excised from gels and purified (section 2.2.7). The TnGV fragments used were *Pst*I –C and -A, *Bam*HI -G, -C and -D, *Xho*I –L and -A, *Bam*HI -A and *Eco*RI –A and -D. Regions of approximately 2.8 kbp between fragments *Bam*HI-D and *Xho*I-L, and 2.6 kbp between *Xho*I-A and *Bam*HI-A were not covered. This was due to the comigration of fragments of a similar size, which made them difficult to isolate.

Approximately 600 ng viral DNA (Table 3.1) was digested with the appropriate restriction enzyme and electrophoresed through a 0.7 % agarose gel, along with approximately 300 ng of *Sal*I-digested cosmid DNA, *Sal*I-digested CpGV DNA or digested TnGV DNA (section 2.2). The gels were Southern blotted and hybridisations were carried out under low stringency (55°C) using either radioactive or DIG-labelled probes (sections 2.5.4 and 2.5.5). Heterologous hybridisation varies considerably at different levels of stringency. Therefore, all of the hybridisations were performed at 55°C under the same salt conditions. Even at minimal stringency, the ability of heterologous DNAs to form stable hybrids is essentially eliminated if their sequences diverge by more than 33 % (Howley *et al.*, 1979). Therefore, any hybridisation detected even at low stringency represents a fairly high degree of similarity.

Representative ethidium bromide-stained gels and autoradiographs are shown along with maps depicting hybridising areas, Figures 3.2-3.21. Restriction fragments under 1.5 kbp, which did not give a visible signal but were within an otherwise hybridising region have been depicted as hybridising for the clarity of the diagrams. This is because these fragments may be too small to give a visible signal at exposure times appropriate

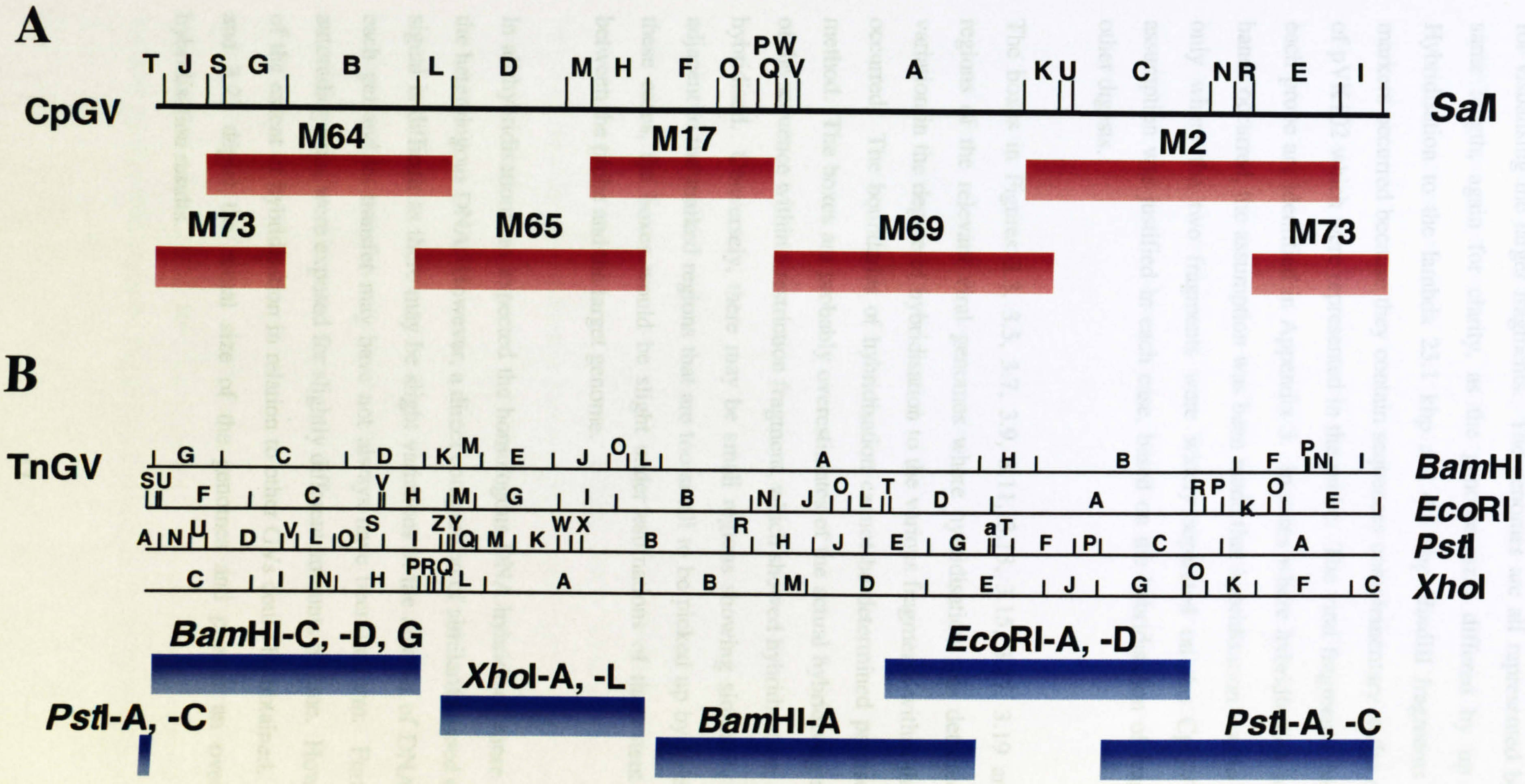


Figure 3.1

A) Cosmids of CpGV (red) and B) fragments of TnGV (blue) to show location of genome fragments used in the hybridisation experiments as probes.

for visualising the larger fragments. The genomes are all represented by lines of the same length, again for clarity, as the genome sizes differed by up to 75 kbp. Hybridisation to the lambda 23.1 kbp and 4.4 kbp *Hin*dIII fragments used as size markers occurred because they contain sequences complementary to the *cos* sequences of pVK102 which were represented in the probe. The viral fragments that hybridise to each probe are identified in Appendix 3. In cases where hybridisation to comigrating bands occurred, the assumption was been made that hybridisation was to one fragment only where the two fragments were widely separated on the CpGV map. This assumption was justified in each case, based on the hybridisation of fragments in the other digests.

The boxes in Figures 3.3, 3.5, 3.7, 3.9, 3.11, 3.13, 3.15, 3.17, 3.19 and 3.21 show regions of the relevant viral genomes where hybridisation was detected. However, variation in the degree of hybridisation to the various fragments within the boxes often occurred. The boundaries of hybridisation cannot be determined precisely using this method. The boxes are probably overestimates of the actual hybridising regions, as all of the sequence within a restriction fragment, which showed hybridisation, may not have hybridised. Conversely, there may be small regions showing similarity in fragments adjacent to the marked regions that are too small to be picked up by hybridisation. In these cases, the boxes would be slight under estimations of the extent of similarity between the probe and the target genome.

In all hybridisations, as expected the homologous DNA hybridised more strongly than the heterologous DNA. However, a direct comparison of similarity based on strength of signal is difficult as there may be slight variations in the amount of DNA loaded onto each gel and its transfer may have not always have been uniform. Furthermore, the autoradiographs were exposed for slightly different amounts of time. However, an idea of the extent of hybridisation in relation to other GVs could be obtained. Figures 3.22 and 3.23 depict the actual size of the genomes and present an overview of the hybridisation results.

3.2.1 ClGV and CpGV (Figures 3.2 and 3.3)

CpGV showed a high degree of similarity to ClGV. It also exhibited collinearity and almost all fragments of ClGV hybridised to CpGV cosmids. There were a few regions where there was no detectable hybridisation such as *Eco*RI-H which has been reported previously (Jehle *et al.*, 1992) and *Nde*I-O and -J. These regions may contain homologous genes that are too dissimilar to be detected by low stringency hybridisation or may contain different genes or DNA. There was another small region that did not hybridise, around *Bam*HI-L of ClGV. This fragment is small as are the corresponding *Eco*RI fragments and so absence of hybridisation could be due to a weak signal that was not detected.

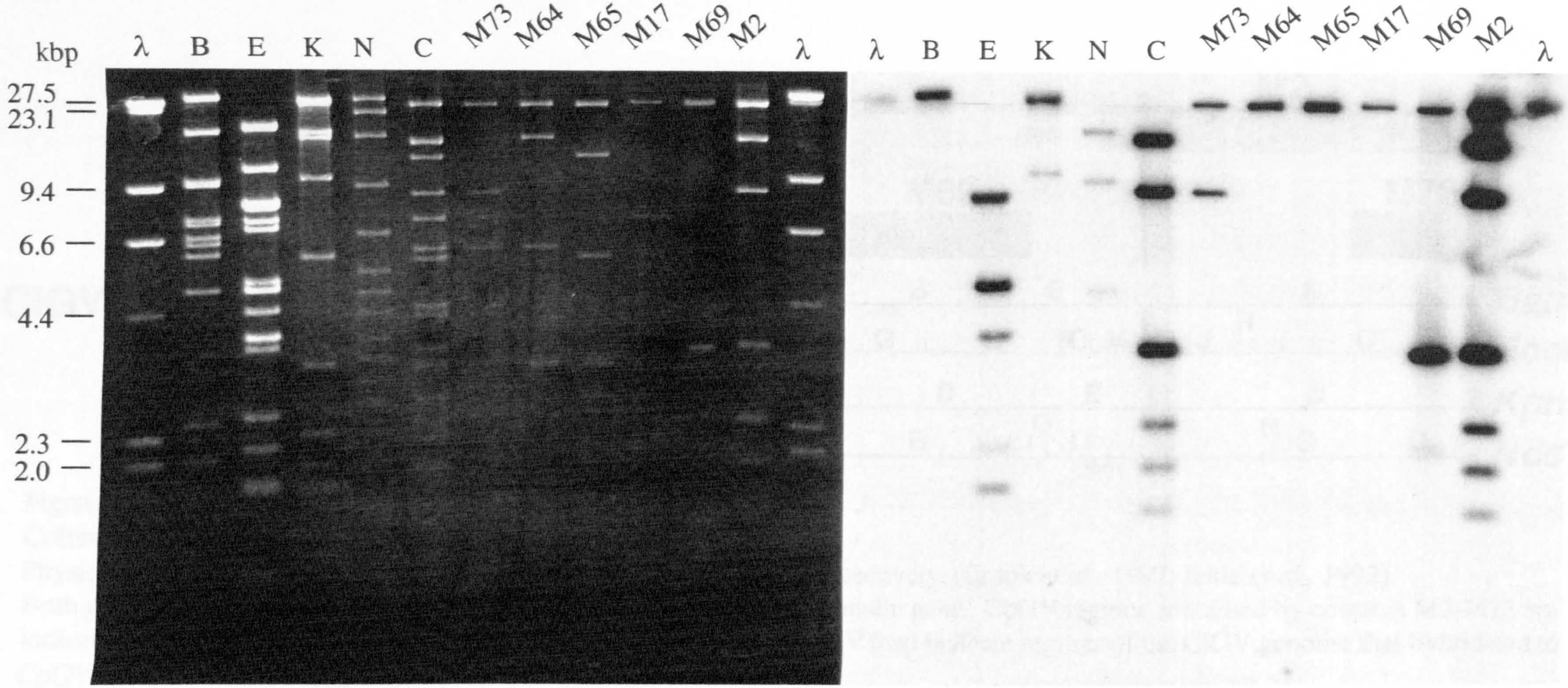
It is not surprising that CpGV and ClGV are so similar since both infect fruit tree tortricid pests. They also have overlapping host ranges with both pests occurring together in certain parts of the world. CpGV can infect *C. leucotreta* as well as *C. pomonella*, although ClGV infects only *C. leucotreta* and not *C. pomonella*. This could be an indication that the hosts are closely related and have recently diverged in the scale of evolution, as could be the case for their respective viruses.

3.2.2 PrGV and CpGV (Figures 3.4 and 3.5)

PrGV displayed less similarity to CpGV than ClGV did to CpGV, although most restriction fragments hybridised to some extent. PrGV infects larvae of the family Pieridae. However, the virus is similar to CpGV in that it is fast to kill and has a small genome of 106.5 kbp. It is clear from these data that these two viruses have collinear organisation. Again there were regions that did not hybridise; in fact there is a large region of about 17 kbp in PrGV that did not hybridise to any CpGV cosmids. This region corresponds to where M64 and M65 overlap on the CpGV genome. This region is *Sal*I-L in CpGV, which contains genes that have no homologues on the database (D. Winstanley and D. O'Reilly, personal communication). The 17 kbp gap in PrGV is a lot larger than CpGV *Sal*I-L, therefore there must be further differences around this region between PrGV and CpGV. Regions of the PrGV genome that hybridise to M64 or M65 are both smaller than expected suggesting that regions in the DNA of these cosmids are

Fig 3.2
Hybridisation of CpGV cosmid M2 to a blot containing restriction enzyme digested ClGV DNA.

Left panel: Gel of digests of ClGV with *Bam*HI (B), *Eco*RI (E), *Kpn*I (K) and *Nde*I (N). *Sal*I digests of CpGV (C) and CpGV cosmids are included. With DNA size ladder λ *Hin*dIII (λ).
Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blot and hybridisation at low stringency to ^{32}P dCTP-labelled CpGV cosmid M2. Hybridising fragments are tabulated in Appendix Table 3.1 together with data from other blots and the data are summarised diagrammatically in Figure 3.3.



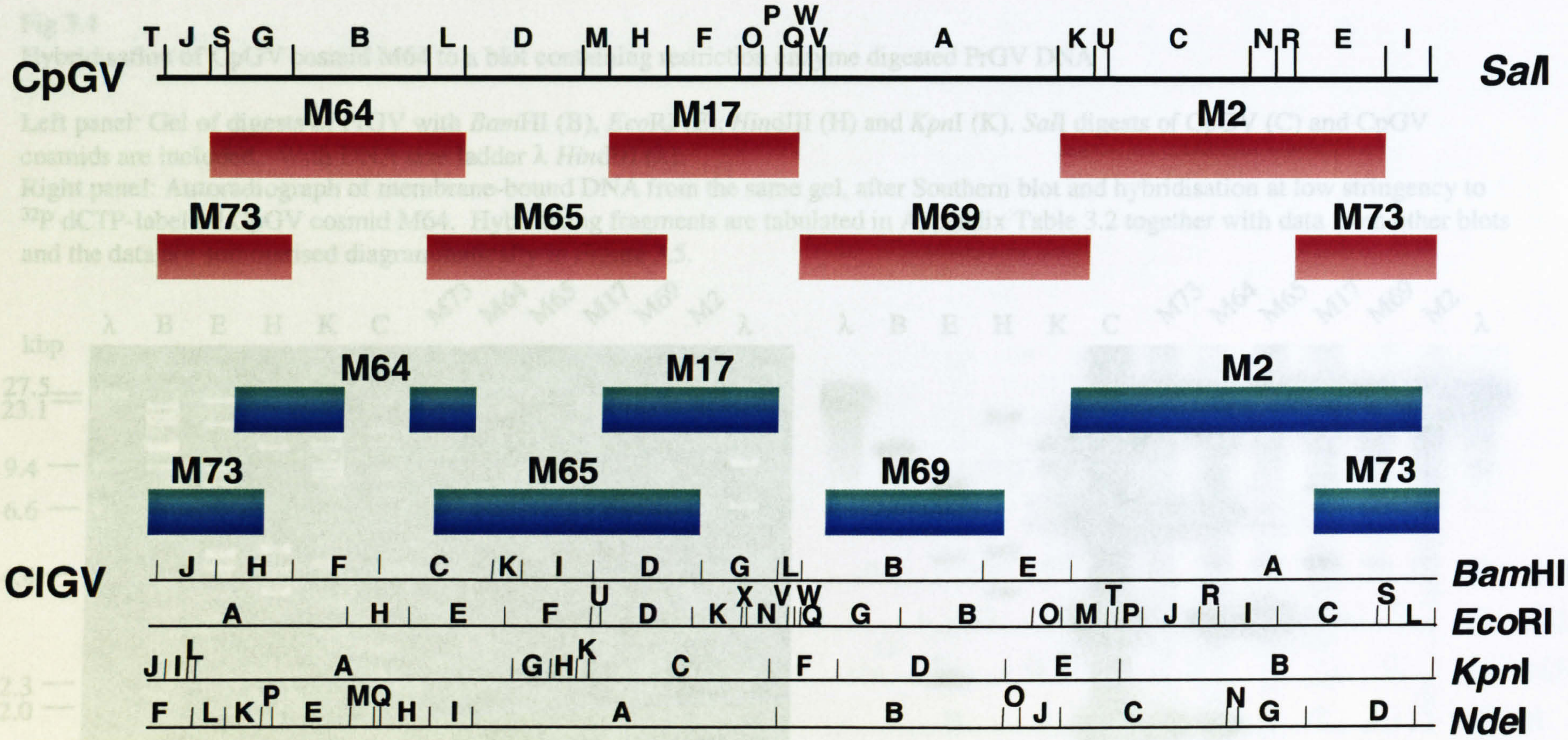
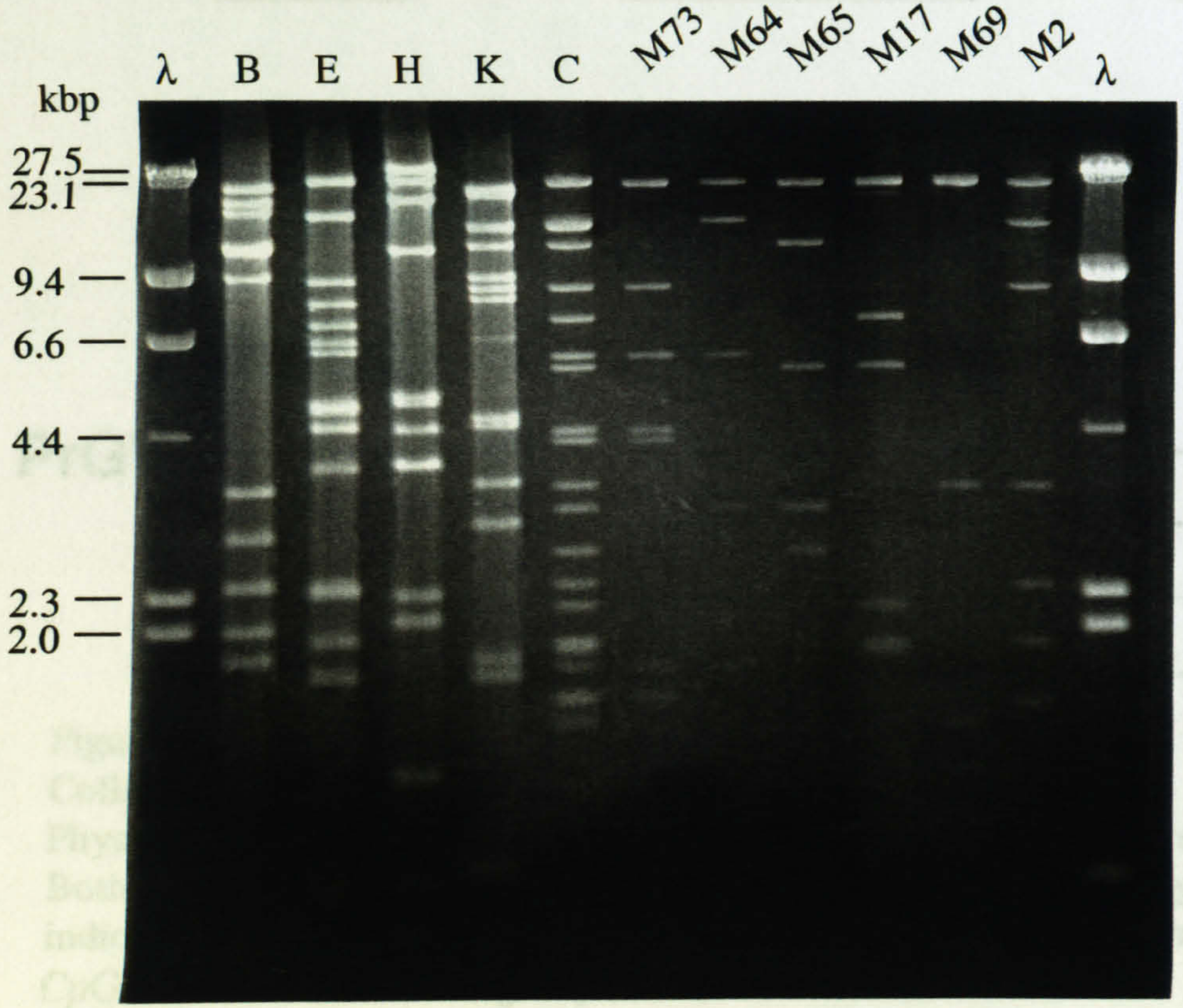
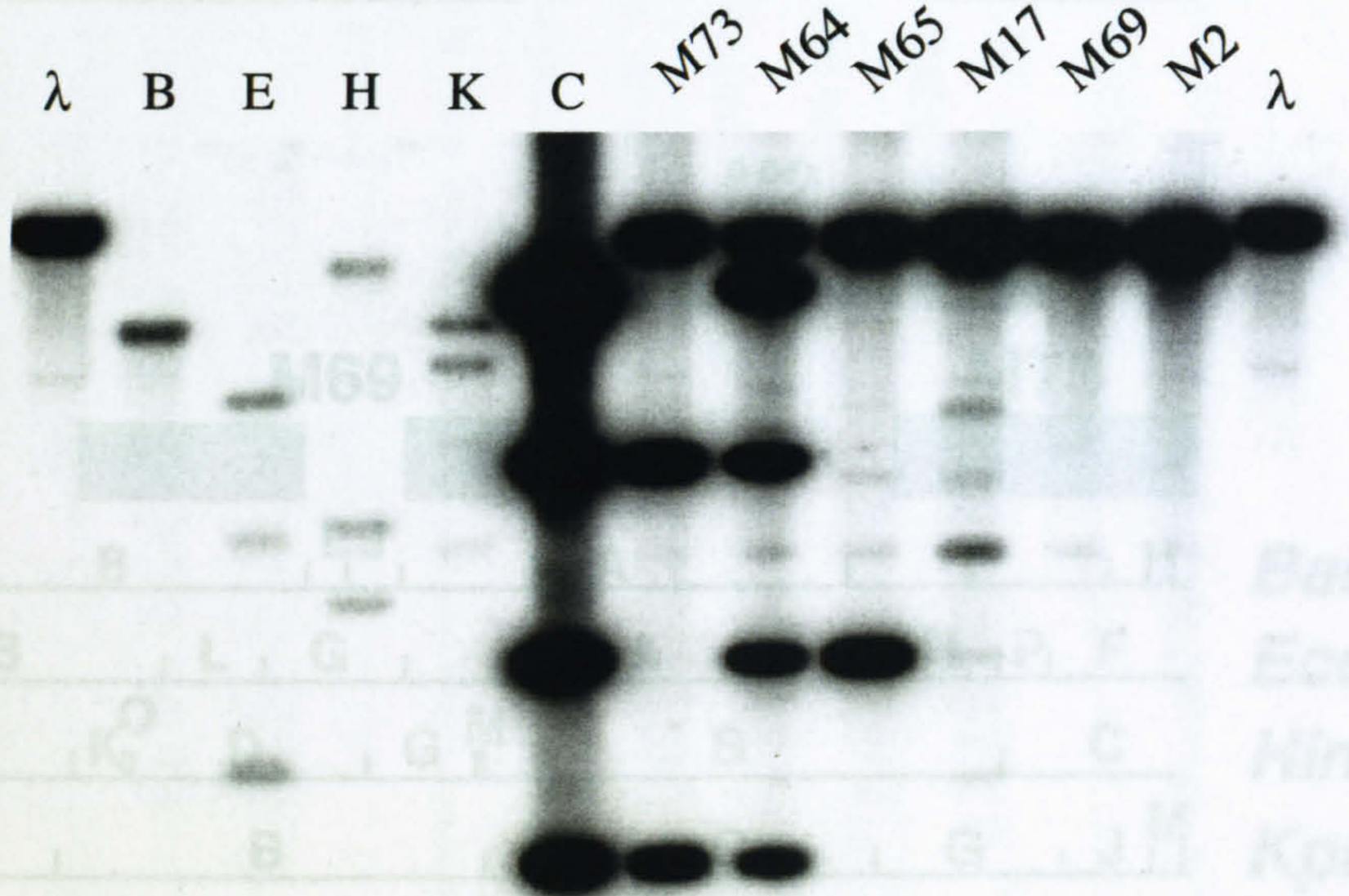


Figure 3.3
Collinearity of CpGV and ClGV genomes.
Physical maps of CpGV and ClGV are shown at the top and bottom respectively. (Crook *et al.*, 1997; Jehle *et al.*, 1992).
Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the ClGV map indicate regions of the ClGV genome that hybridised to CpGV cosmids at low stringency (55°C).

Fig 3.4
Hybridisation of CpGV cosmid M64 to a blot containing restriction enzyme digested PrGV DNA

Left panel: Gel of digests of PrGV with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Kpn*I (K). *Sal*I digests of CpGV (C) and CpGV cosmids are included. With DNA size ladder λ *Hin*dIII (λ).
Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blot and hybridisation at low stringency to ^{32}P dCTP-labelled CpGV cosmid M64. Hybridising fragments are tabulated in Appendix Table 3.2 together with data from other blots and the data are summarised diagrammatically in Figure 3.5.





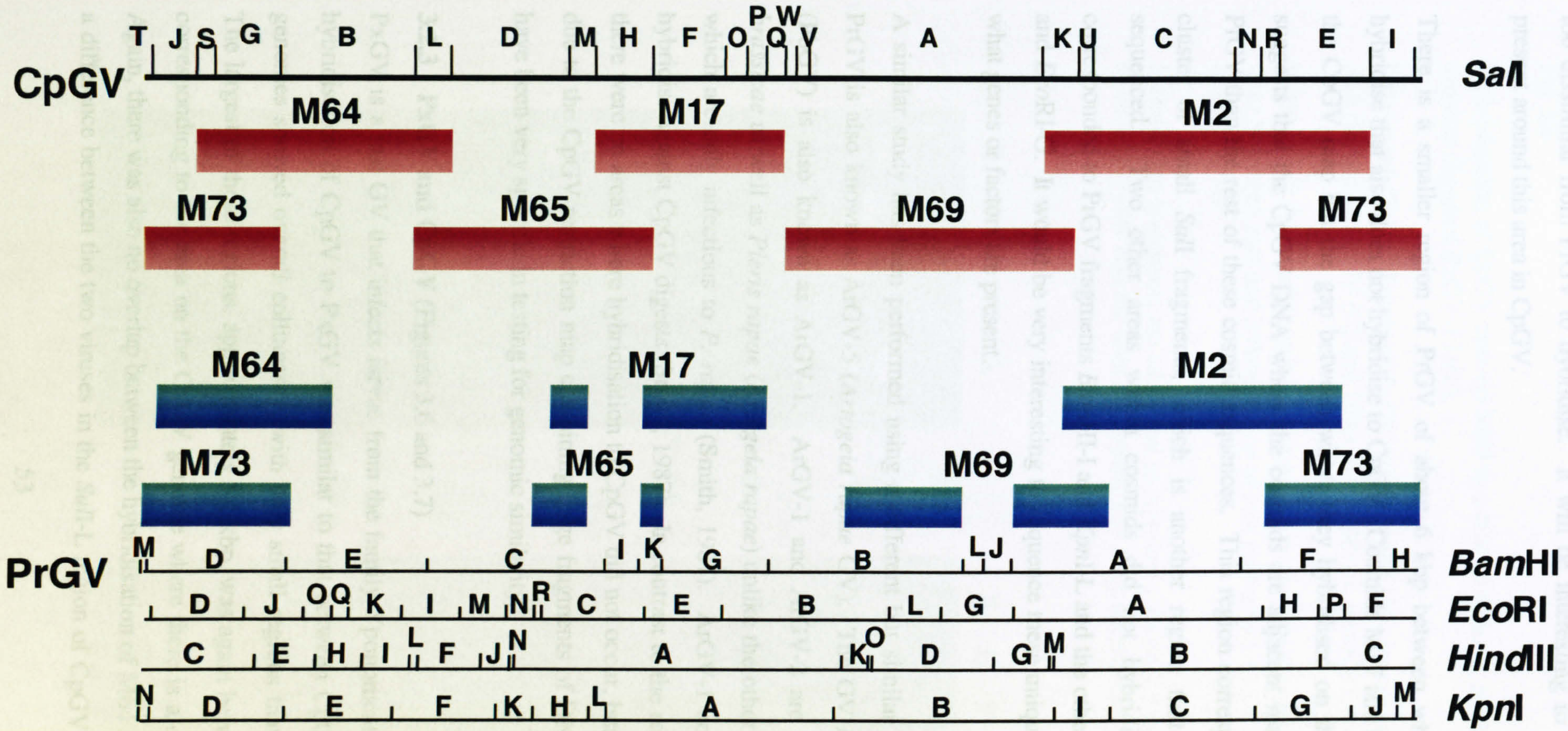


Figure 3.5
Collinearity of CpGV and PrGV genomes.
Physical maps of CpGV and PrGV are shown at the top and bottom respectively. (Crook *et al.*, 1997; Dwyer and Granados, 1987). Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the PrGV map indicate regions of the PrGV genome that hybridised to CpGV cosmids at low stringency (55°C).

too dissimilar from PrGV to hybridise. It will be interesting to see what genes are present around this area in CpGV.

There is a smaller region of PrGV of about 6 kbp between where M17 and M69 hybridise that also does not hybridise to CpGV. Cosmids M17 and M69 are adjacent on the CpGV map but the gap between where they hybridised on the PrGV map again suggests that the CpGV DNA where the cosmids are adjacent may be less similar to PrGV than the rest of these cosmid sequences. This region corresponds in CpGV to a cluster of small *Sal*I fragments, which is another region that is currently being sequenced. Two other areas within cosmids did not hybridise. One of these corresponded to PrGV fragments *Bam*HI-I and *Kpn*I-L and the other to *Bam*HI-L and -J and *Eco*RI-G. It would be very interesting to sequence these unique areas to determine what genes or factors are present.

A similar study has been performed using a different but similar genotype of PrGV. PrGV is also known as ArGV-5 (*Artogeia rapae* GV). The GV *Pieris brassicae* GV (PbGV) is also known as ArGV-1. ArGV-1 and ArGV-2 are infectious to *Pieris brassicae* as well as *Pieris rapae* (*Artogeia rapae*) unlike the other genotypes of ArGV which are only infectious to *P. rapae* (Smith, 1987). ArGV-1 cosmids were used to hybridise against CpGV digests (Smith, 1987). In contrast to the results described here, there were no areas where hybridisation to CpGV did not occur, but this may have been due to the CpGV restriction map containing large fragments of DNA which would not have been very specific in testing for genomic similarity.

3.2.3 PxGV and CpGV (Figures 3.6 and 3.7)

PxGV is a fast GV that infects larvae from the family Yponomeutidae. The extent of hybridisation of CpGV to PxGV was similar to that between CpGV and PrGV. The genomes showed overall collinearity, with only small regions that did not hybridise. The largest of these regions, approximately 5.1 kbp, was again between M17 and M69, corresponding to the area on the CpGV genome where there is a cluster of *Sal*I sites. Again, there was also no overlap between the hybridisation of M64 and M65, suggesting a difference between the two viruses in the *Sal*I-L region of CpGV. There was also no

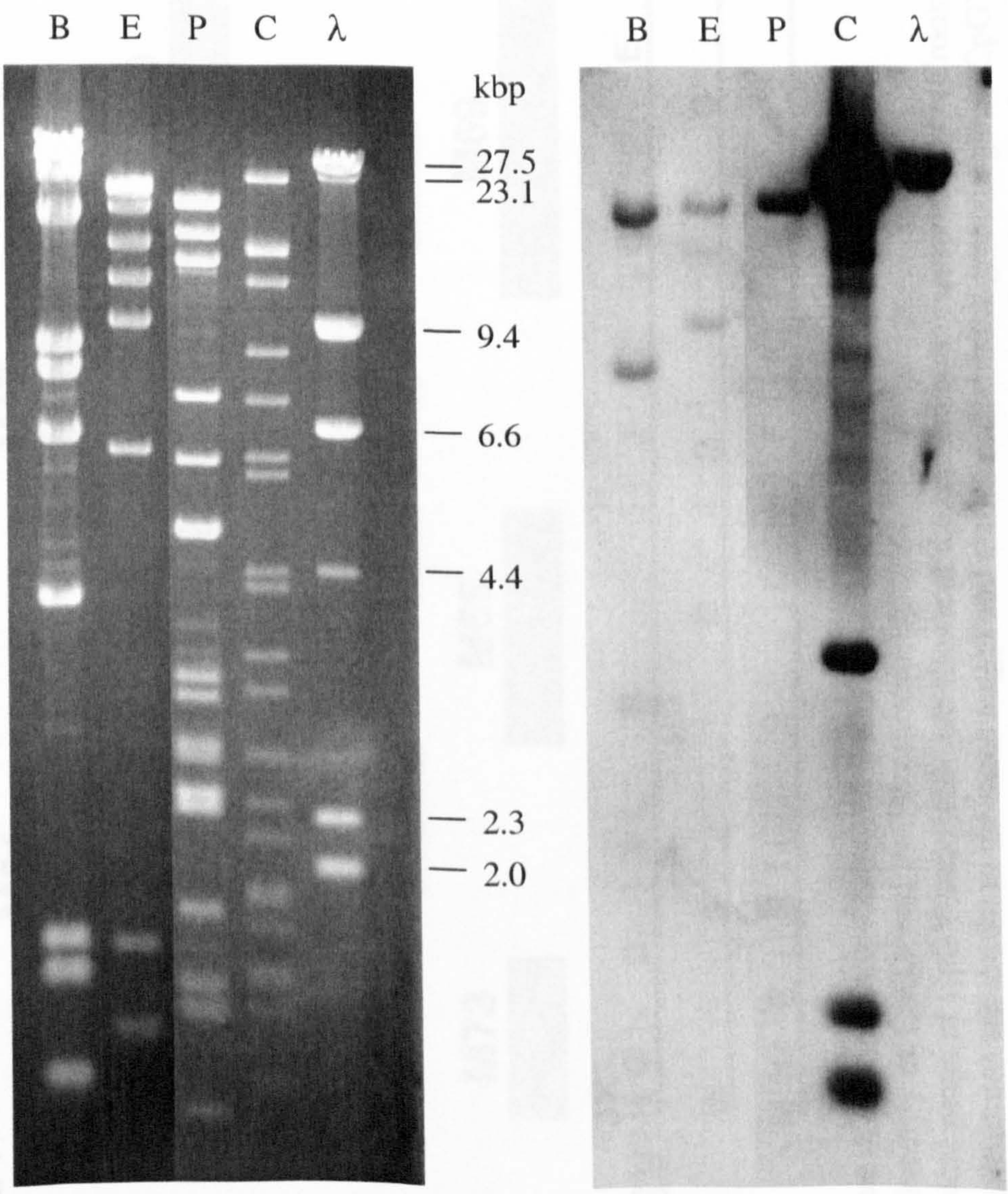
Fig 3.6

Hybridisation of CpGV cosmid M69 to a blot containing restriction enzyme digested PxGV-J DNA

Left panel: Gel of digests of PxGV with *Bam*HI (B), *Eco*RI (E) and *Pst*I (P). *Sal*I digests of CpGV (C) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blot and hybridisation at low stringency to DIG labelled CpGV cosmid M69.

Hybridising fragments are tabulated in Appendix Table 3.3 together with data from other blots and the data is summarised diagrammatically in Figure 3.7.



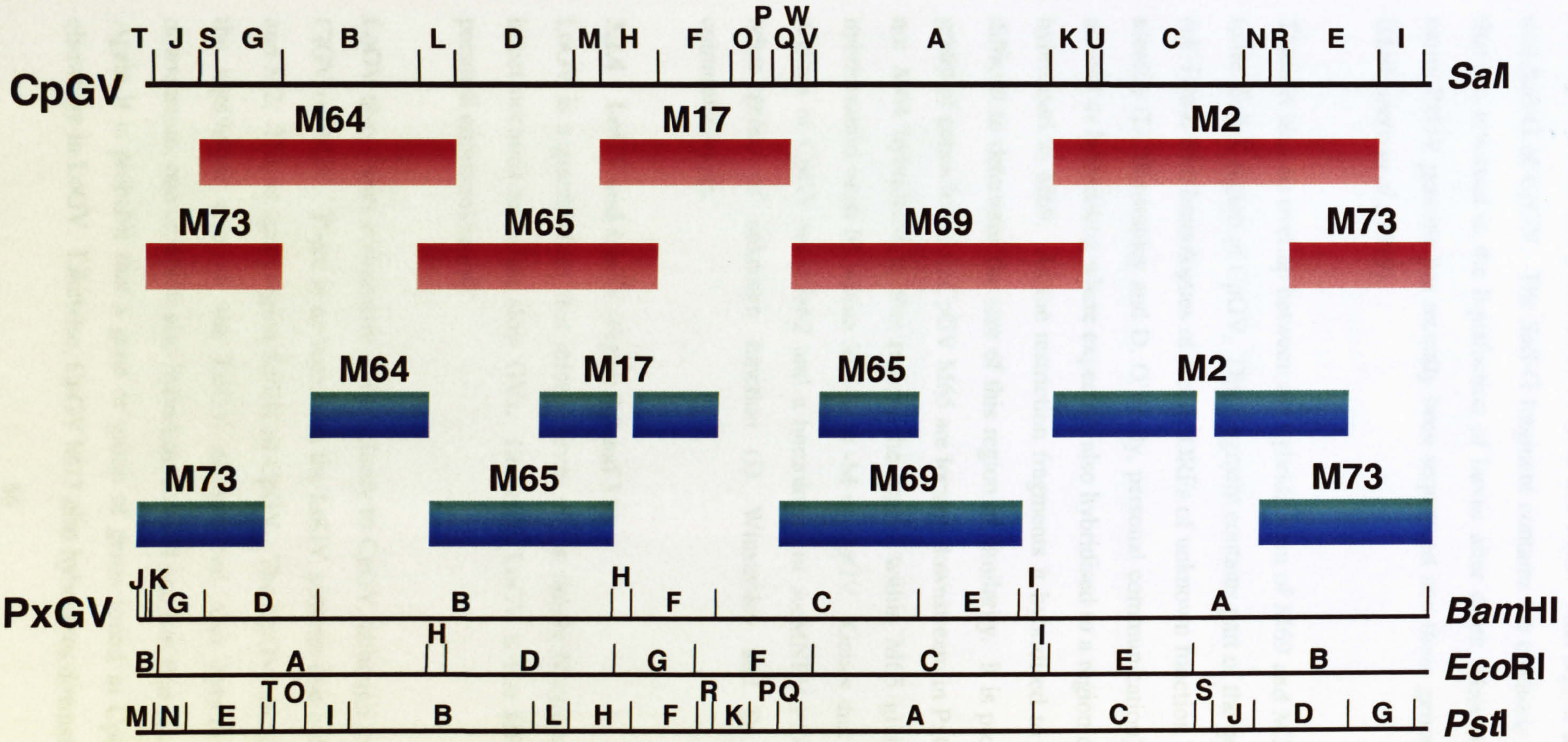


Figure 3.7
Collinearity of CpGV and PxGV genomes.
Physical maps of CpGV and PxGV are shown at the top and bottom respectively. (Crook *et al.*, 1997; Hashimoto *et al.*, 2000). Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the PxGV map indicate regions of the PxGV genome that hybridised to CpGV cosmids at low stringency (55°C).

overlap between the hybridisation of M73 and M64. This region corresponds to *Sal*I-S and *Sal*I-G of CpGV. The *Sal*I-G fragment contains the chitinase and cathepsin genes that are involved in the liquefaction of larvae after death (Hawtin *et al.*, 1997). The entire PxGV genome has recently been sequenced and these genes were not identified (Hashimoto *et al.*, 1999).

There is also no overlap between the hybridisation of M69 and M2, which corresponds to the *Sal*I-K region of CpGV. This fragment contains part of the gene *very late factor 1* (*vlf-1*) and four homologues of XcGV ORFs of unknown function with low amino acid identity (D. Winstanley and D. O'Reilly, personal communication). The cosmid M65, as well as hybridising where expected, also hybridised to a region downstream that also hybridised to M69. As the restriction fragments it hybridised to are quite large, it is difficult to determine the size of this region of similarity. It is probable that a gene or group of genes found in CpGV M65 are located downstream in PxGV. As neither M17 nor M64 hybridised to this region, the region within M65 giving this downstream hybridisation must be within *Sal*I-D or -M of CpGV. Genes that were found in these regions of CpGV were *lef-2* and a homologue of AcMNPV PDV-E66 gene, among other genes of unknown function (D. Winstanley and D. O'Reilly, personal communication).

3.2.4 LoGV and CpGV (Figure 3.8 and 3.9)

LoGV is a granulovirus that infects larvae of the family Noctuidae. The viruses that infect noctuids are often slow GVs. However, LoGV is fast killing (I. R. L. Smith, personal communication).

LoGV also shows collinearity and similarity to CpGV, although less complete than for ClGV or PrGV. There is no region on the LoGV genome that hybridises to both M69 and M2. This is again region *Sal*I-K of CpGV. The CpGV cosmid M65 hybridises to the equivalent area on the LoGV genome but also hybridises to two regions downstream, one of which also hybridises to M64 and one that also hybridises to M73. Again it is probable that a gene or group of genes found in CpGV M65 are located elsewhere in LoGV. Likewise, CpGV M73 also hybridises downstream to LoGV *Xho*I-

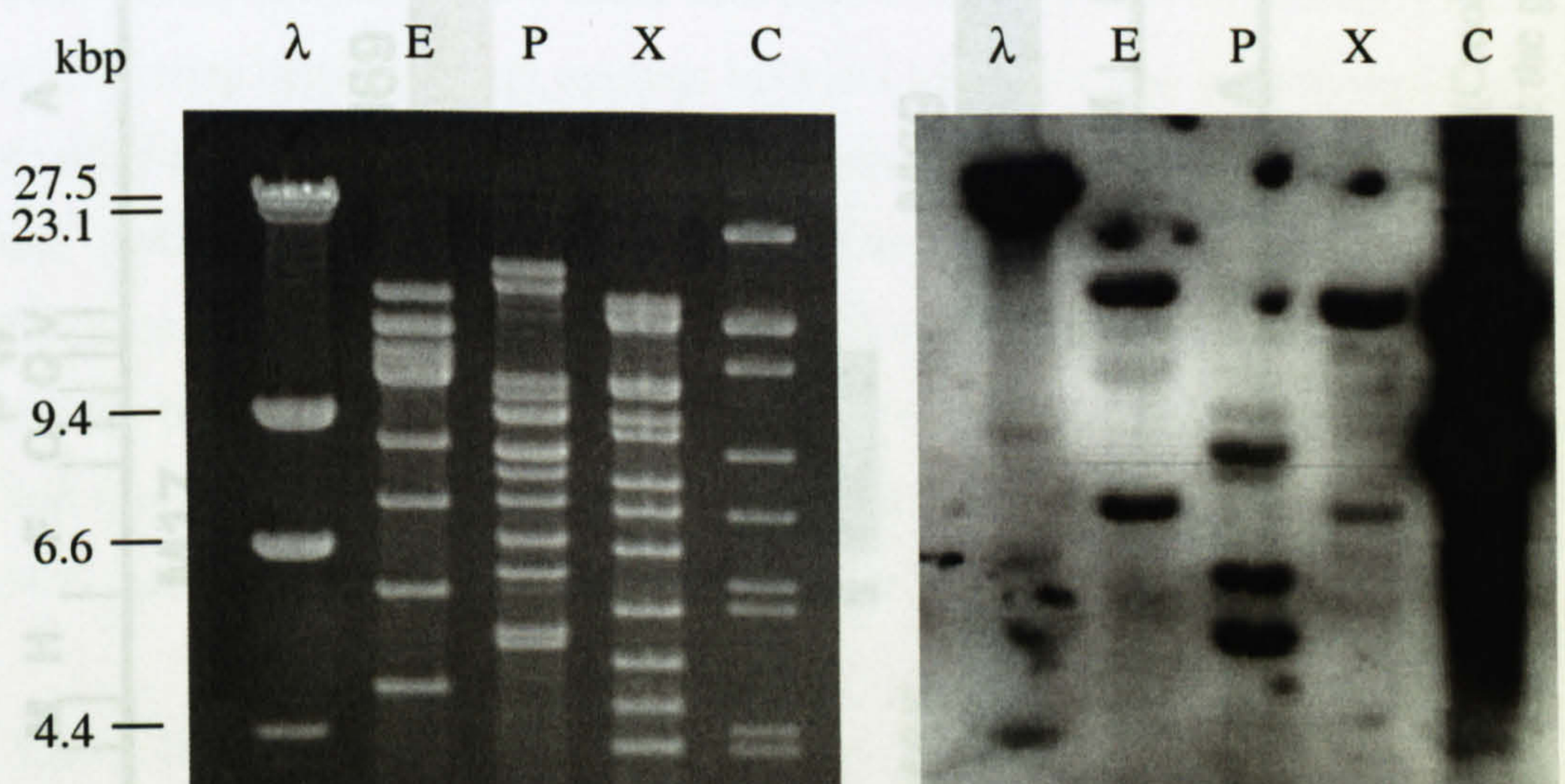
Fig 3.8

Hybridisation of CpGV cosmid M2 to a blot containing restriction enzyme digested LoGV-S DNA

Left panel: Gel of digests of LoGV with *Eco*RI (E), *Pst*I (P) and *Xho*I (X). *Sal*I digests of CpGV (C) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blot and hybridisation at low stringency to DIG labelled CpGV cosmid M2.

Hybridising fragments are tabulated in Appendix Table 3.4 together with data from other blots and the data are summarized diagrammatically in Figure 3.9.



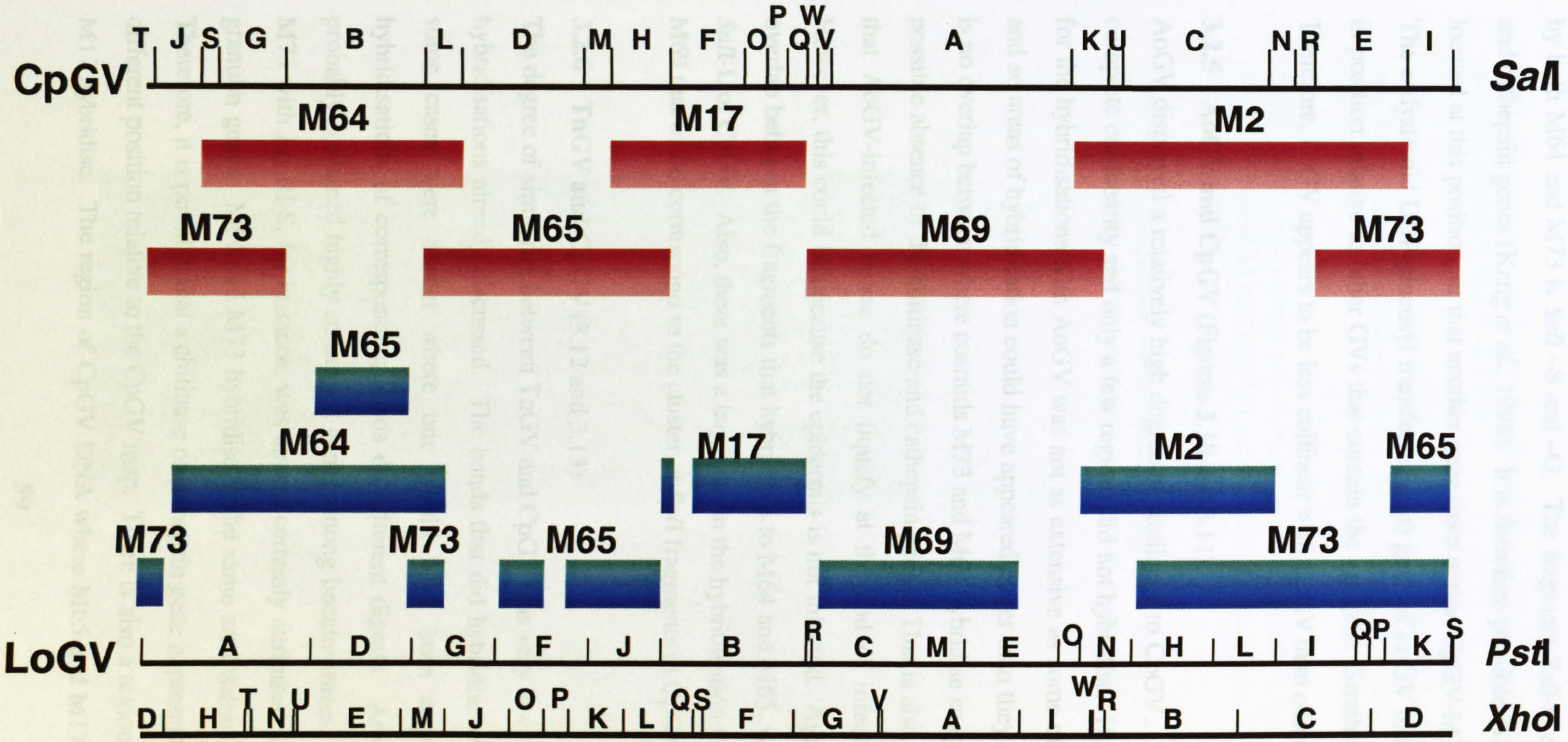


Figure 3.9
Collinearity of CpGV and LoGV genomes.
Physical maps of CpGV and LoGV are shown at the top and bottom respectively (Crook *et al.*, 1997; I. R. L. Smith, personal communication). Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the LoGV map indicate regions of the LoGV genome that hybridised to CpGV cosmids at low stringency (55°C).

M, which also hybridises to M64. The region of the CpGV genome, which is contained by both M64 and M73 is *Sal*I –S and –G. The fragment *Sal*I-G contains the chitinase and cathepsin genes (Kang *et al.*, 1998). It is therefore possible that these genes may be located at this position, or that another gene from within CpGV-M73 is in that position. The ecdysteroid UDP-glucosyl transferase (*egt*) gene of LoGV has been found to differ in position relative to other GVs that contain the *egt* gene (Smith and Goodale, 1998). Therefore, LoGV appears to be less collinear with CpGV than other fast-killing GVs.

3.2.5 AoGV and CpGV (Figures 3.10 and 3.11)

AoGV displayed a relatively high degree of similarity to CpGV. The genomes showed complete collinearity and only a few regions did not hybridise. However, the map used for the hybridisations with AoGV was not as extensive as some of the other maps used and so areas of hybridisation could have appeared larger than they actually were. There is no overlap between where cosmids M73 and M64 hybridise suggesting once again the possible absence of the chitinase and cathepsin genes. This is also supported by the fact that AoGV-infected larvae do not liquefy at the end of infection (section 4.2.5). However, this could be because the epidermis is not infected. Again, there was also no overlap between the fragments that hybridised to M64 and M65, which corresponded to *Sal*I-L of CpGV. Also, there was a large gap in the hybridisation map between M17 and M69 that could correspond to the cluster of *Sal*I fragments in CpGV.

3.2.6 TnGV and CpGV (3.12 and 3.13)

The degree of similarity between TnGV and CpGV was very low compared to the other hybridisations already discussed. The bands that did hybridise were very faint and in some cases were absent where one would have been expected based on the hybridisations of corresponding bands on different digests. Areas that did hybridise probably contained highly conserved genes among baculoviruses. The hybridisation of M73 with *Eco*RI-S, for instance, was almost certainly attributed to hybridisation of the granulin genes. M64 and M73 hybridise to the same area within *Bam*HI–J of TnGV. Therefore, it is possible that a chitinase or cathepsin gene is present within this area, in a different position relative to the CpGV map. There is also a region where both M65 and M17 hybridise. The region of CpGV DNA where M65 and M17 overlap contains the

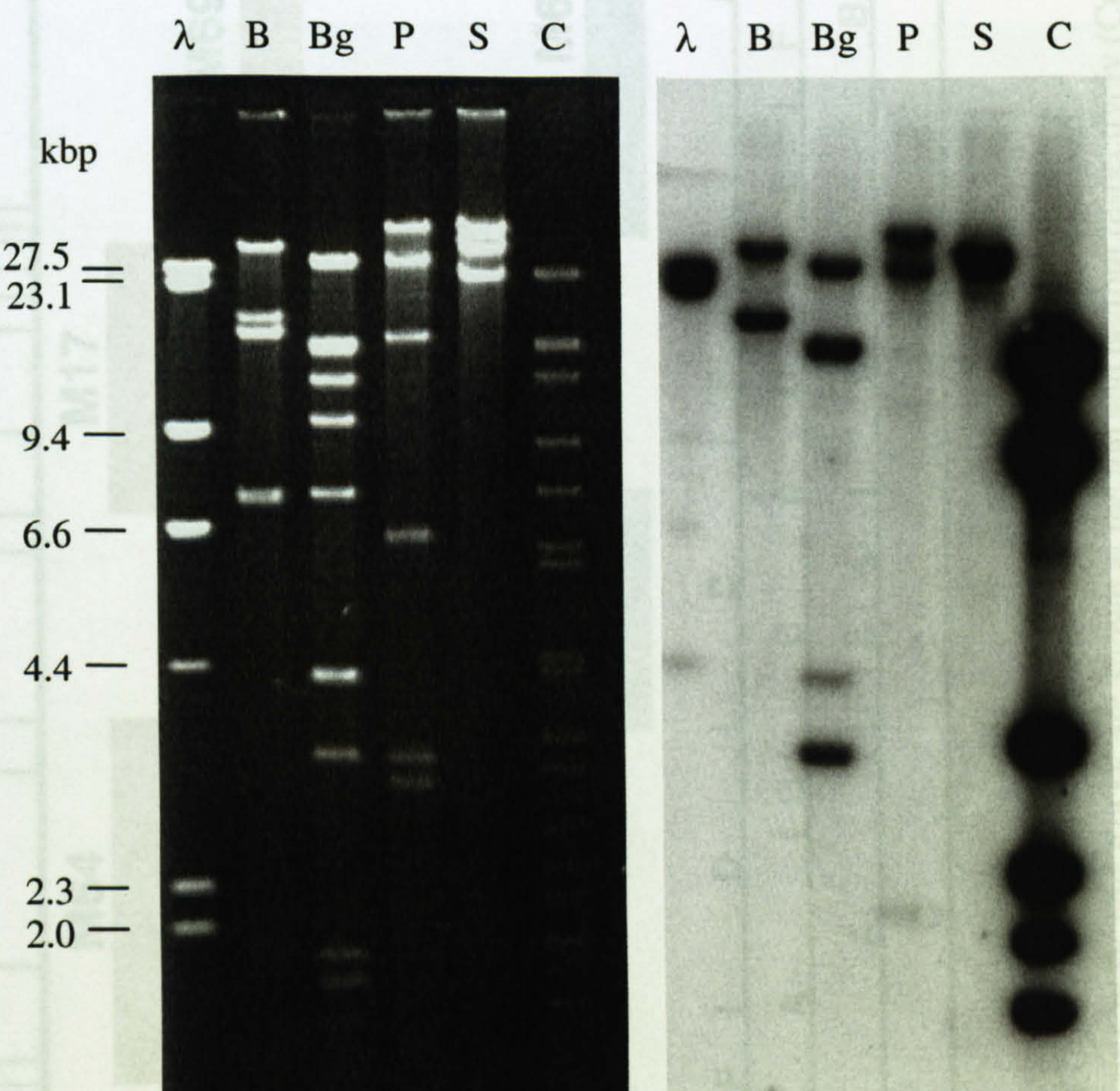
Fig 3.10

Hybridisation of CpGV cosmid M2 to a blot containing restriction enzyme digested AoGV-E DNA

Left panel: Gel of digests of AoGV with *Bam*HI (B), *Bgl*II (Bg), *Pst*I (P) and *Sac*I (S). *Sal*I digests of CpGV (C) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blot and hybridisation at low stringency to ^{32}P dCTP-labelled CpGV cosmid M2.

Hybridising fragments are tabulated in Appendix Table 3.5 together with data from other blots and the data are summarised diagrammatically in Figure 3.11.



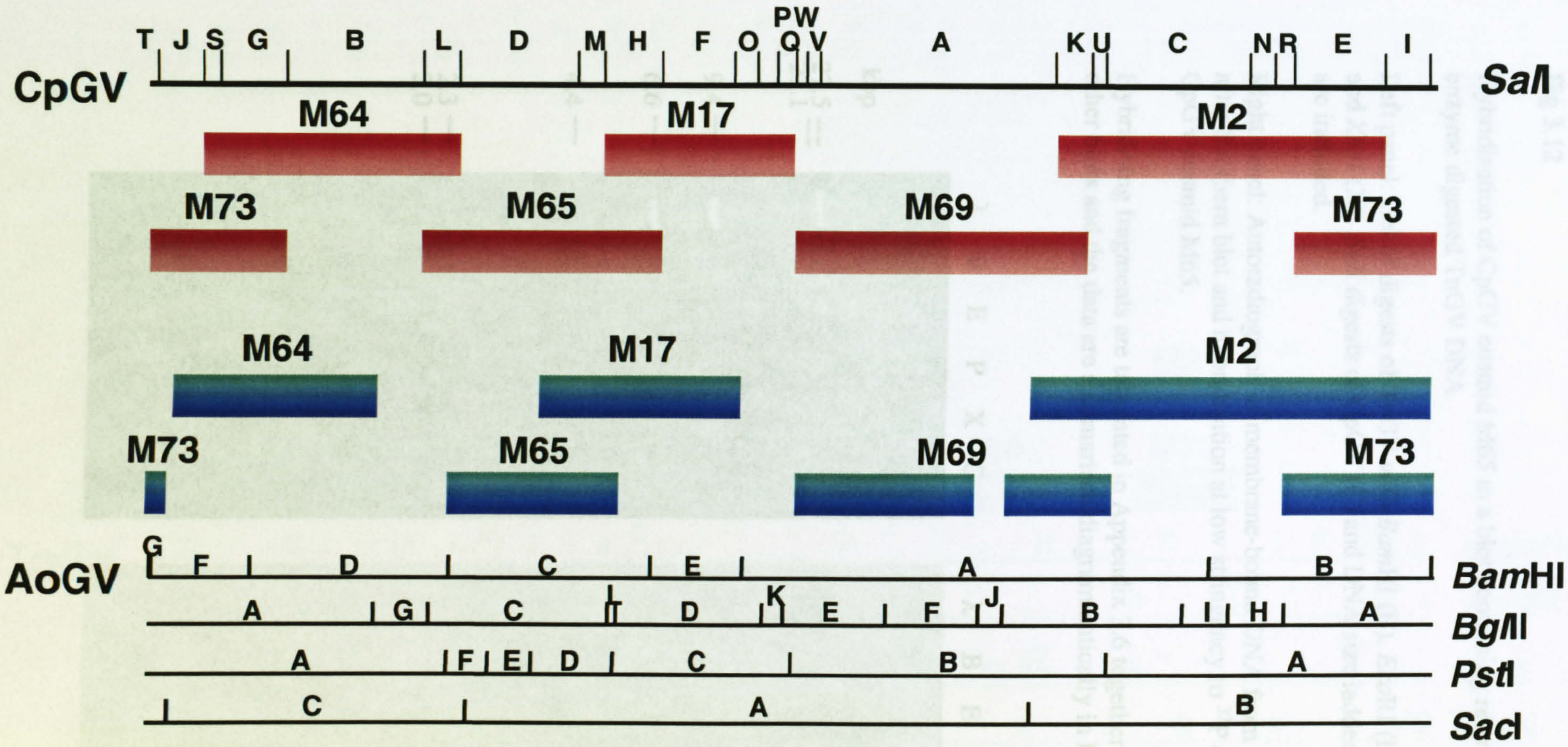


Figure 3.11
Collinearity of CpGV and AoGV genomes.
Physical maps of CpGV and AoGV are shown at the top and bottom respectively (Crook *et al.*, 1997; Figure 4.16).
Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the AoGV map indicate regions of the AoGV genome that hybridised to CpGV cosmids at low stringency (55°C).

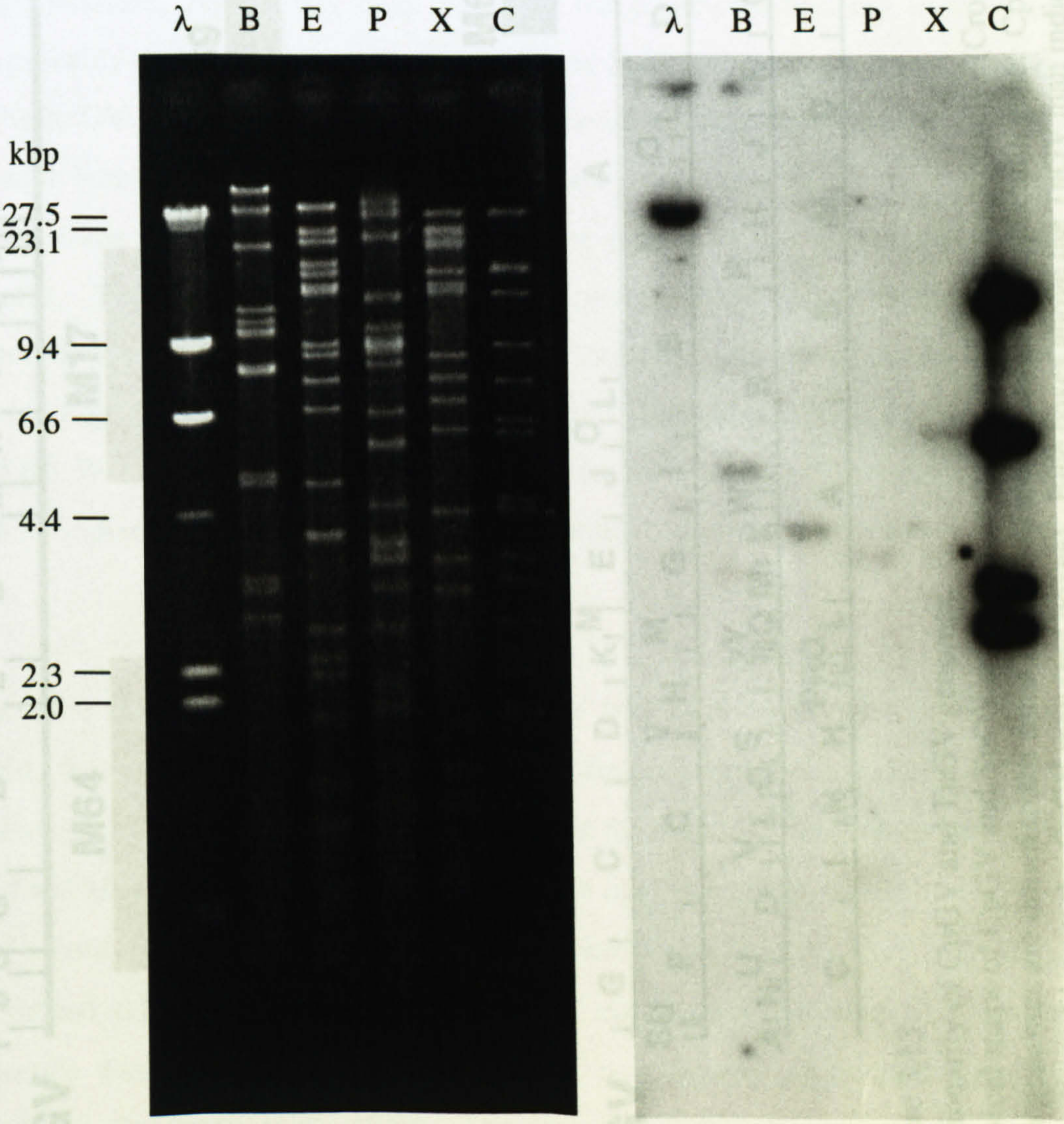
Fig 3.12

Hybridisation of CpGV cosmid M65 to a blot containing restriction enzyme digested TnGV DNA

Left panel: Gel of digests of TnGV with *Bam*HI (B), *Eco*RI (E), *Pst*I (P) and *Xho*I (X). *Sal*I digests of CpGV (C) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blot and hybridisation at low stringency to ^{32}P dCTP-labelled CpGV cosmid M65.

Hybridising fragments are tabulated in Appendix 3.6 together with data from other blots and the data are summarised diagrammatically in Figure 3.13.



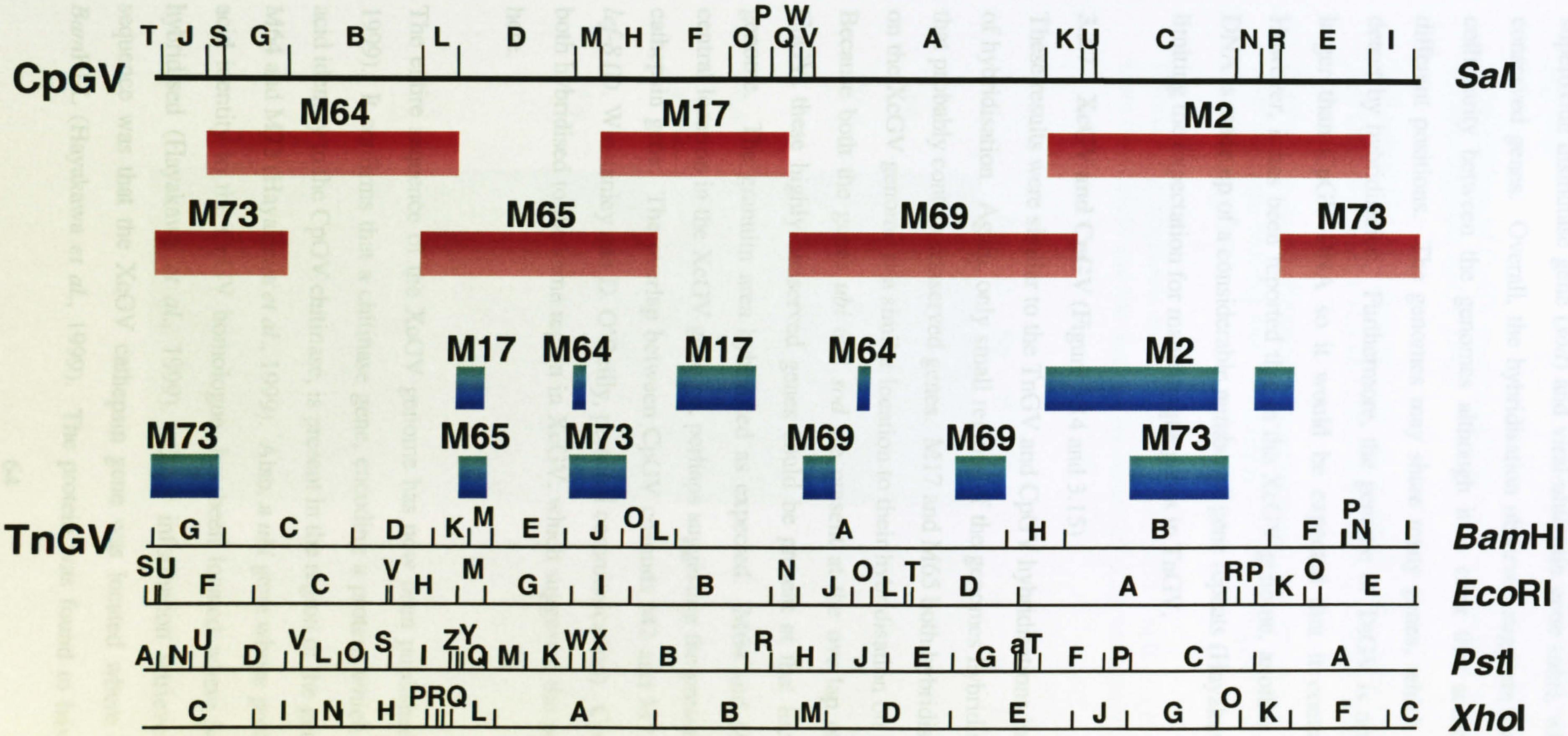


Figure 3.13
Collinearity of CpGV and TnGV genomes.
Physical maps of CpGV and TnGV are shown at the top and bottom respectively. (Crook *et al.*, 1997; Hashimoto *et al.*, 1996). Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the TnGV map indicate regions of the TnGV genome that hybridised to CpGV cosmids at low stringency (55°C).

superoxide dismutase gene (*sod*) and viral-ubiquitin gene (*ubi*), which are both highly conserved genes. Overall, the hybridisation observed suggests that there is a general collinearity between the genomes although it is clear that some genes must be in different positions. The genomes may share many genes, which are too different to detect by hybridisation. Furthermore, the genome of TnGV is approximately 50 kbp larger than CpGV DNA so it would be expected that it contains unique genes. However, it has been reported that in the XcGV genome, another large GV, the extra DNA is made up of a considerable number of gene repeats (Hayakawa *et al.*, 1999) thus limiting the expectation for many unique genes in TnGV.

3.2.7 XcGV and CpGV (Figure 3.14 and 3.15)

These results were similar to the TnGV and CpGV hybridisations in regard to the extent of hybridisation. Again, only small regions of the genomes hybridised, indicating areas that probably contain conserved genes. M17 and M65 both hybridised to a small region on the XcGV genome at a similar location to their hybridisation on the TnGV genome. Because both the genes *ubi* and *sod* are present at the overlap of M17 and M65 in CpGV, these highly conserved genes could be present at that location in the XcGV genome. The granulin area hybridised as expected. M64 and M73 hybridised at a central location in the XcGV genome, perhaps suggesting the presence of a chitinase or cathepsin gene. The overlap between CpGV cosmids M2 and M73 contains the gene *lef-8* (D. Winstanley and D. O'Reilly, personal communication). Cosmids M2 and M73 both hybridised to the same region in XcGV, which suggested the presence of this gene here.

The entire sequence of the XcGV genome has now been published (Hayakawa *et al.*, 1999). It confirms that a chitinase gene, encoding a protein which shows 62% amino acid identity to the CpGV chitinase, is present in the region of the genome hybridising to M64 and M73 (Hayakawa *et al.*, 1999). Also, a *ubi* gene whose protein has 83% amino acid identity to the CpGV homologue, has been located where M17 and M65 both hybridised (Hayakawa *et al.*, 1999). Other information retrieved from the XcGV sequence was that the XcGV cathepsin gene was located where M64 hybridised to *Bam*HI-L (Hayakawa *et al.*, 1999). The protein was found to have 50% amino acid

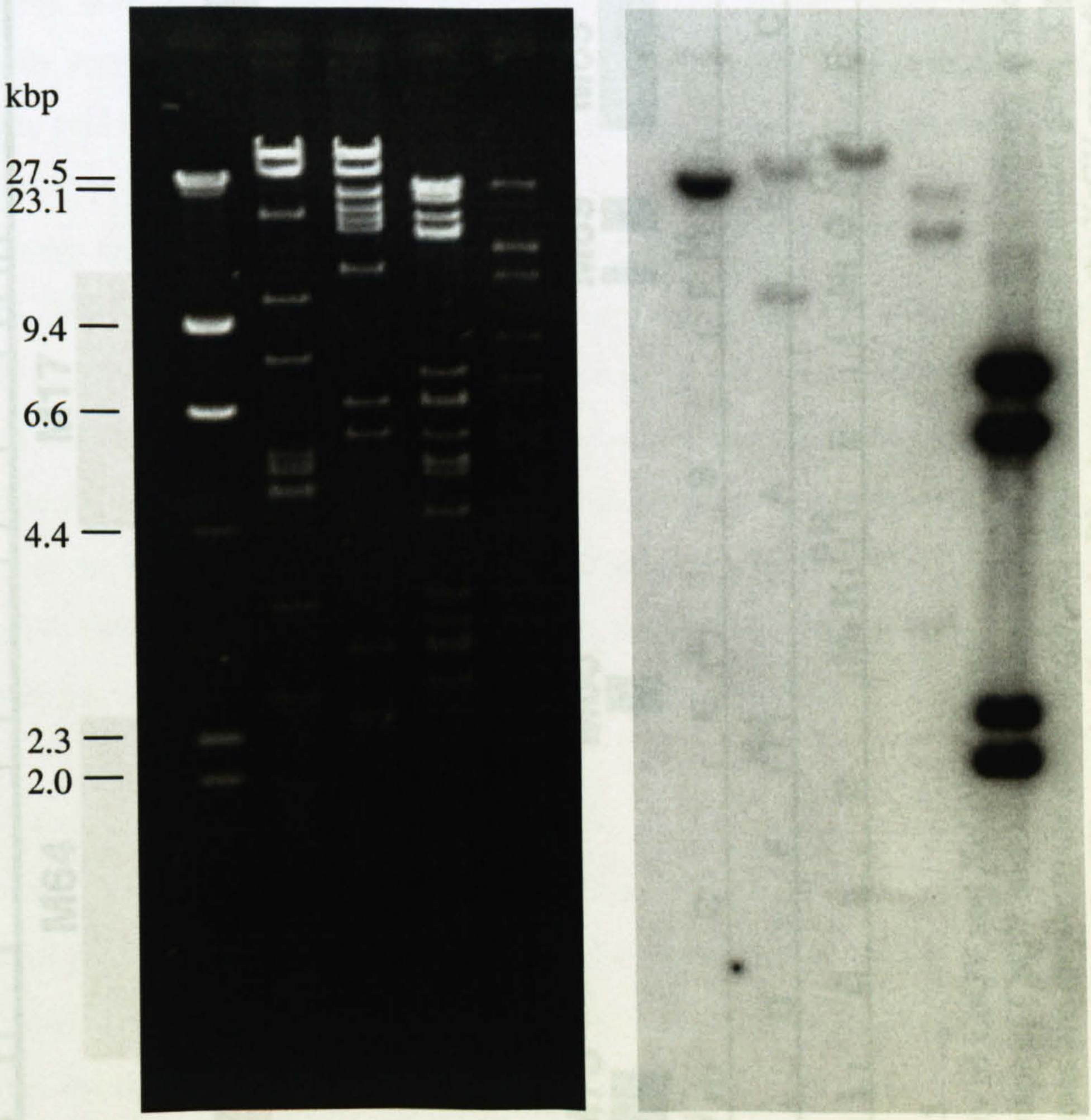
Fig 3.14

Hybridisation of CpGV cosmid M17 to a blot containing restriction enzyme digested XcGV DNA

Left panel: Gel of digests of XcGV with *Bam*HI (B), *Bgl*II (Bg) and *Eco*RI (E). *Sal*I digests of CpGV (C) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blotting and hybridisation at low stringency to ^{32}P dCTP-labelled CpGV cosmid M17.

Hybridising fragments are tabulated in Appendix Table 3.7 together with data from other blots and the data are summarised diagrammatically in Figure 3.15.



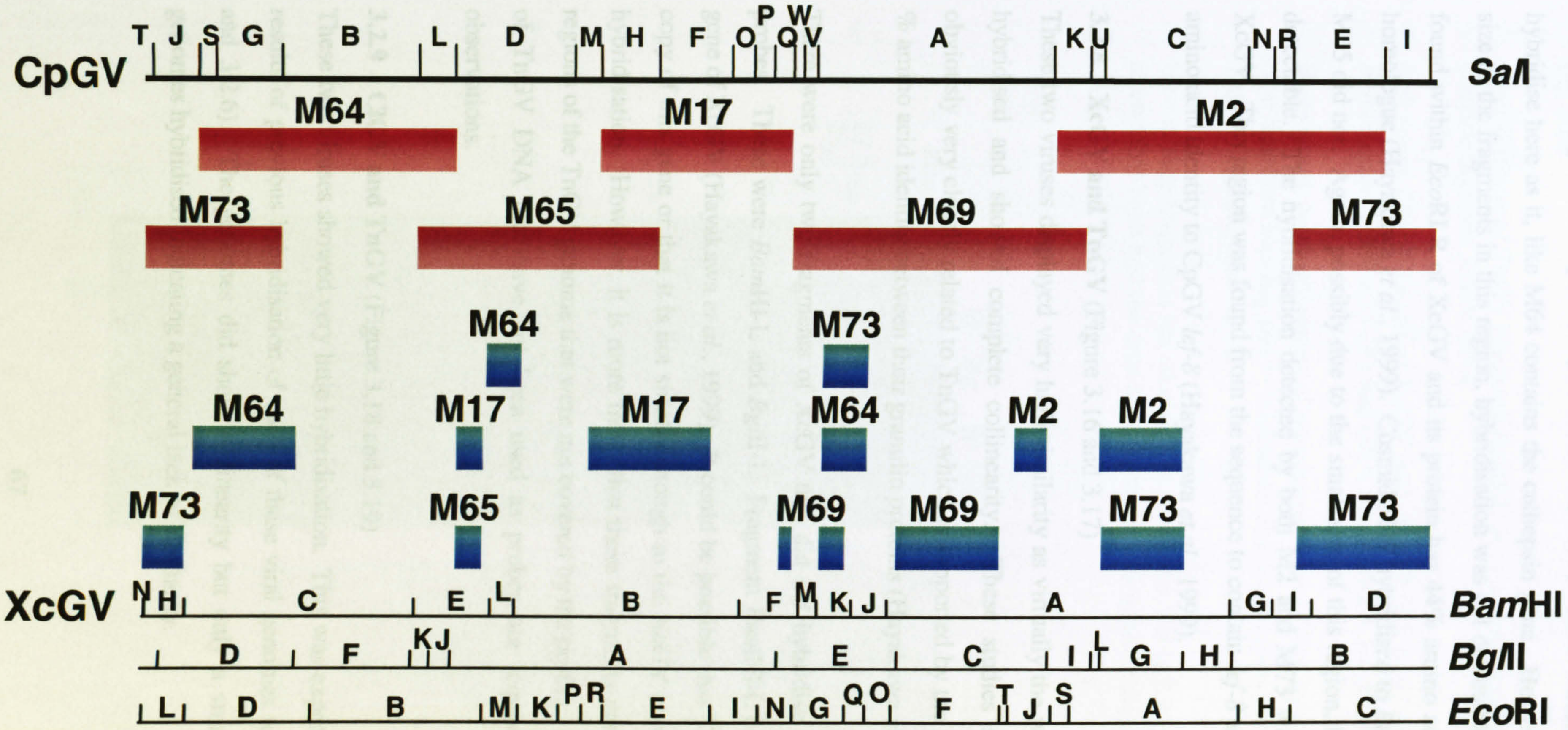


Figure 3.15
Collinearity of CpGV and XcGV genomes.
Physical maps of CpGV and XcGV are shown at the top and bottom respectively (Crook *et al.*, 1997; Goto *et al.*, 1992).
Both genomes are drawn to the same length and are linearised at the granulin gene. CpGV regions contained by cosmids M2-M73 are indicated below the CpGV map (red boxes). Blue boxes above the XcGV map indicate regions of the XcGV genome that hybridised to CpGV cosmids at low stringency (55°C).

identity to CpGV cathepsin (Hayakawa *et al.*, 1999). M73 would also be expected to hybridise here as it, like M64 contains the cathepsin gene. However, due to the small size of the fragments in this region, hybridisation was not detectable. The gene *sod* was found within *Eco*RI-R of XcGV and its protein has 44% amino acid identity to CpGV homologue (Hayakawa *et al.*, 1999). Cosmid M17 hybridised to *Eco*RI-R of XcGV, but M65 did not. Again, possibly due to the small size of this region, hybridisation was not detectable. The hybridisation detected by both M2 and M73 was within *Bgl*II-G of XcGV. This region was found from the sequence to contain *lef-8* as predicted with 64% amino acid identity to CpGV *lef-8* (Hayakawa *et al.*, 1999).

3.2.8 XcGV and TnGV (Figure 3.16 and 3.17)

These two viruses displayed very high similarity as virtually the whole of the genomes hybridised and showed complete collinearity. These studies show that XcGV is obviously very closely related to TnGV which is supported by the fact that there is 99.6 % amino acid identity between their granulin proteins (Hayakawa *et al.*, 1999).

There were only two fragments of XcGV that did not hybridise to any of the TnGV probes. These were *Bam*HI-L and *Bgl*II-J. Fragment *Bam*HI-L contains the cathepsin gene of XcGV (Hayakawa *et al.*, 1999). It could be possible that TnGV does not have a copy of this gene or that it is not similar enough to the XcGV copy to be picked up by hybridisation. However, it is more likely that these fragments correspond to the small regions of the TnGV genome that were not covered by the probes. Probes of the regions of TnGV DNA that have not been used as probes are required to confirm these observations.

3.2.9 ClGV and TnGV (Figure 3.18 and 3.19)

These two viruses showed very little hybridisation. This was expected judging from the results of previous hybridisation of each of these viral genomes to CpGV DNA (3.2.1 and 3.2.6). The genomes did show collinearity but only a small proportion of the genomes hybridised indicating a general lack of similarity.

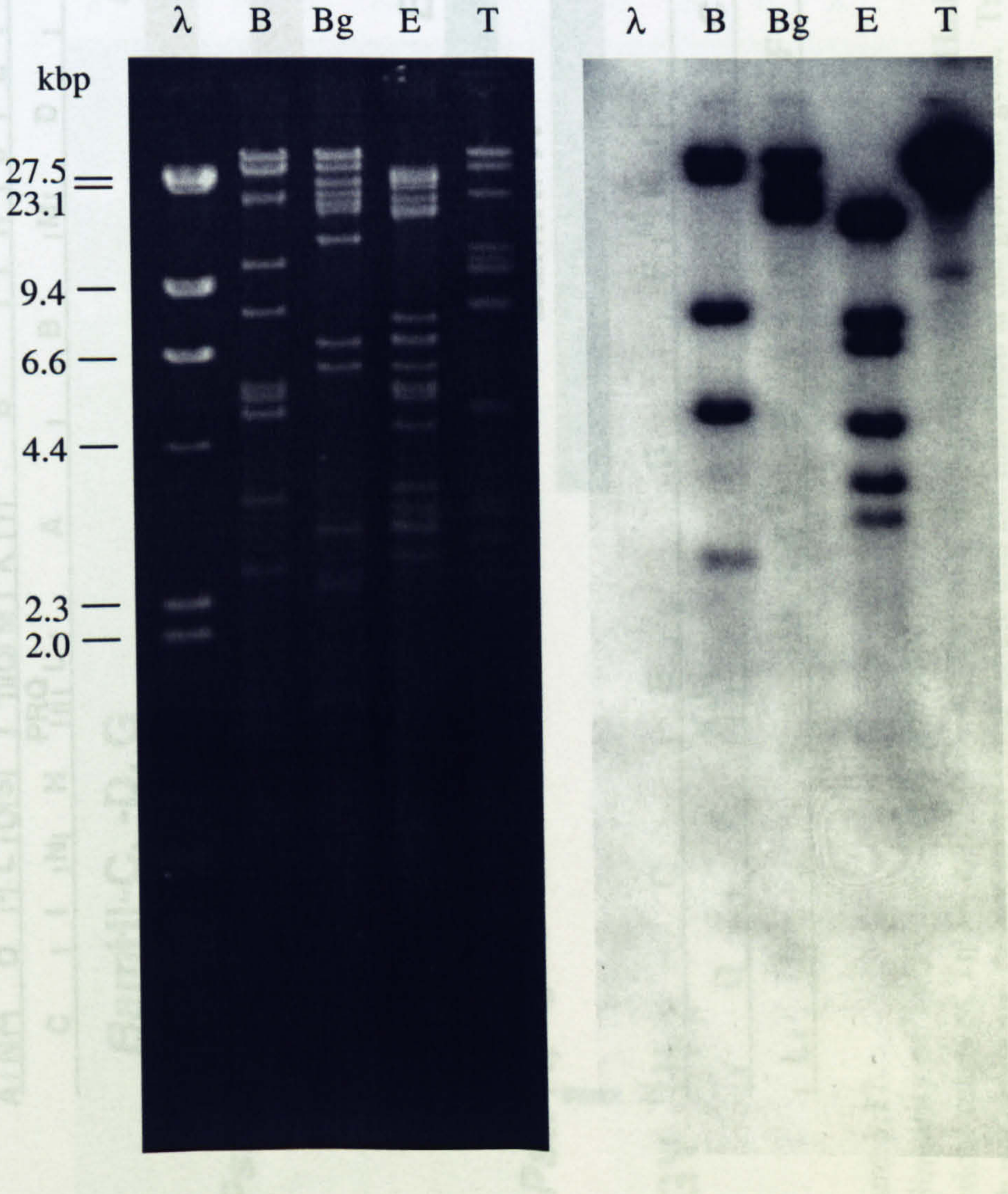
Fig 3.16

Hybridisation of TnGV fragment *Bam*HI -A to a blot containing restriction enzyme digested XcGV DNA

Left panel: Gel of digests of XcGV with *Bam*HI (B), *Bgl*II (Bg) and *Eco*RI (E). *Bam*HI digests of TnGV (T) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blotting and hybridisation at low stringency to ^{32}P dCTP-labelled TnGV fragment *Bam*HI -A.

Hybridising fragments are tabulated in Appendix Table 3.8 together with data from other blots and the data are summarised diagrammatically in Figure 3.17.



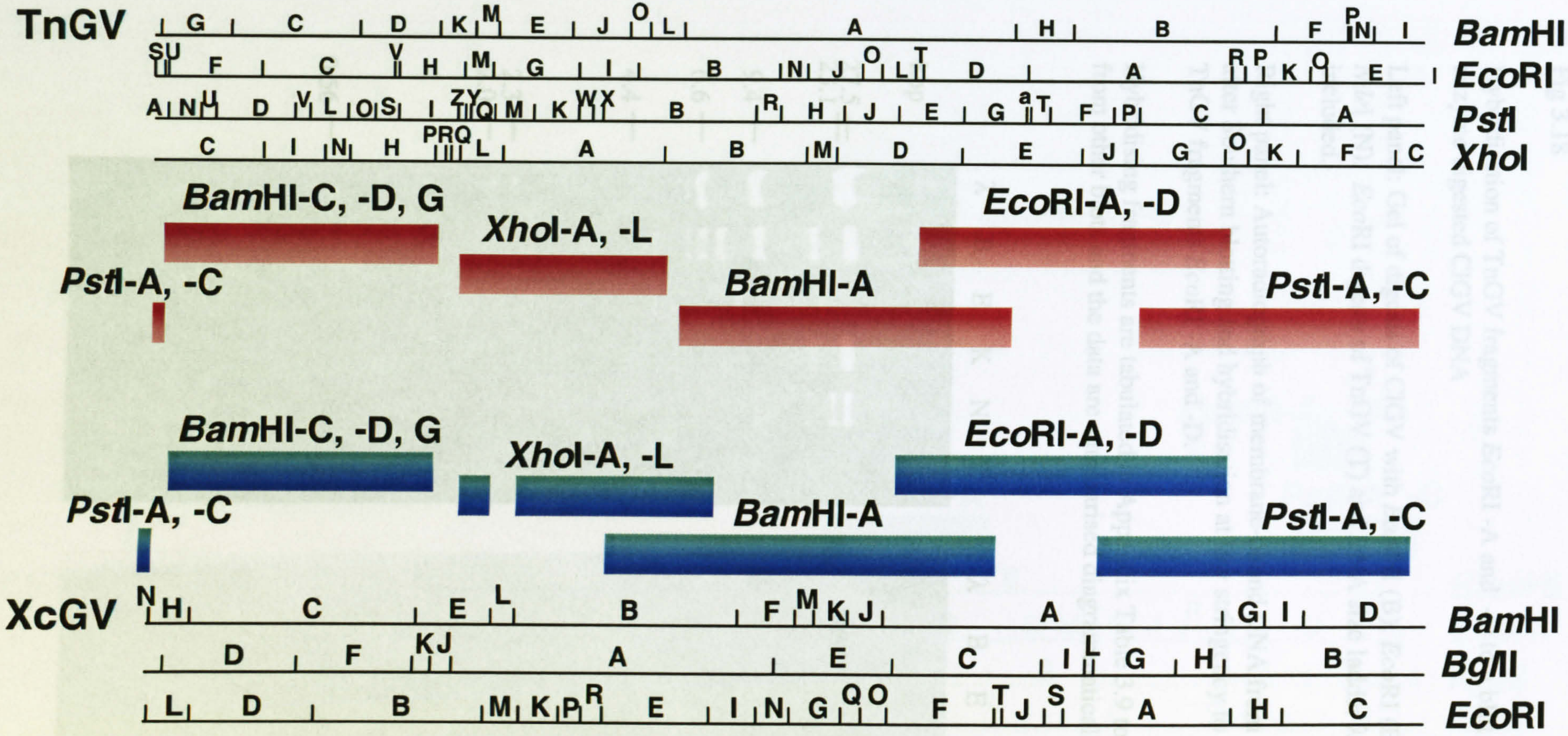


Figure 3.17
Collinearity of TnGV and XcGV genomes.
Physical maps of TnGV and XcGV are shown at the top and bottom respectively (Hashimoto *et al.*, 1996; Goto *et al.*, 1992). Both genomes are drawn to the same length and are linearised at the granulin gene. TnGV fragments of DNA are indicated below the TnGV map (red boxes). Blue boxes above the XcGV map indicate regions of the XcGV genome that hybridised to TnGV fragments of DNA at low stringency (55°C).

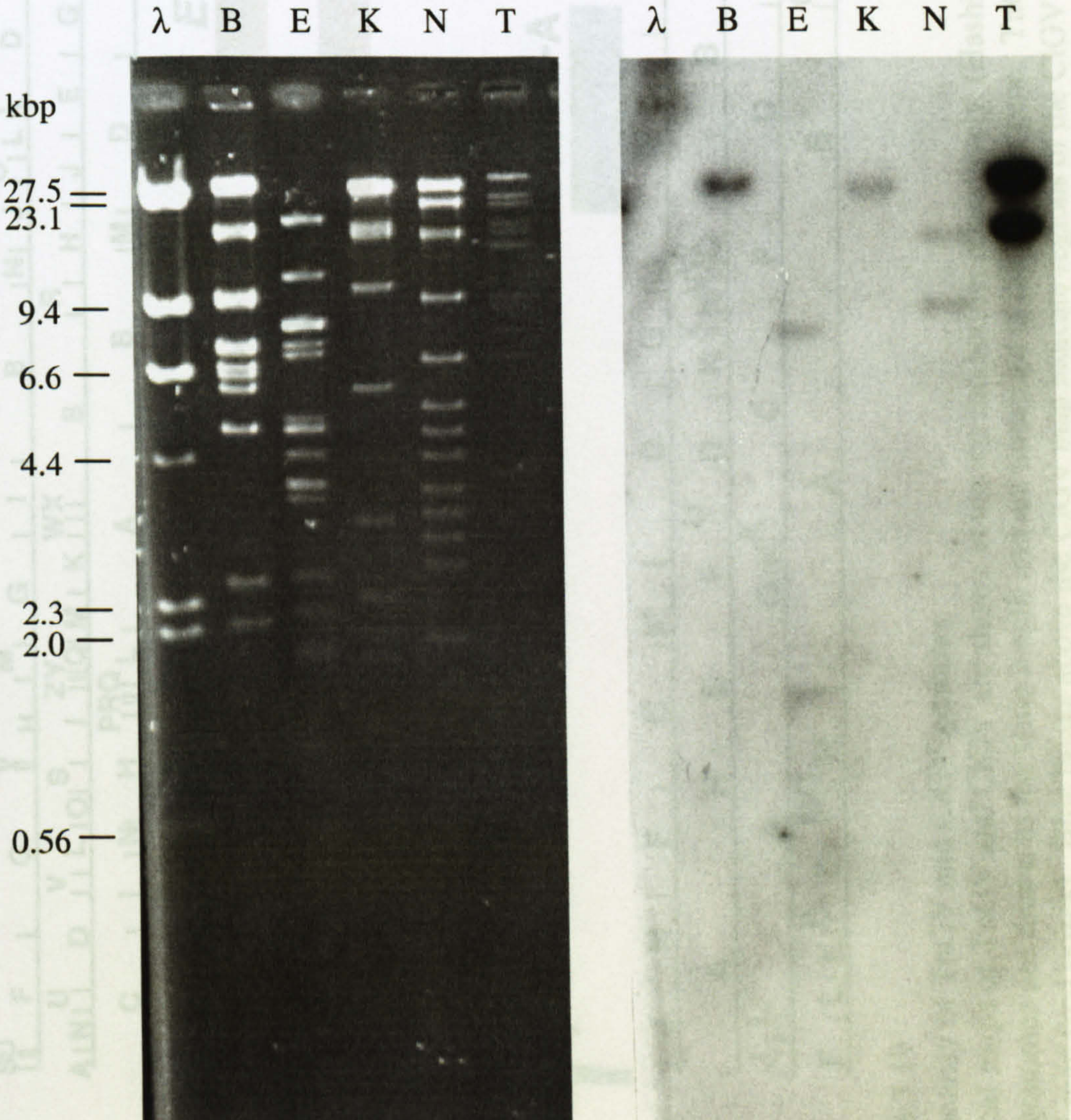
Fig 3.18

Hybridisation of TnGV fragments *Eco*RI -A and -D to a blot containing restriction enzyme digested ClGV DNA

Left panel: Gel of digests of ClGV with *Bam*HI (B), *Eco*RI (E), *Kpn*I (K) and *Nde*I (N). *Eco*RI digests of TnGV (T) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blotting and hybridisation at low stringency to ^{32}P dCTP-labelled TnGV fragments *Eco*RI -A and -D.

Hybridising fragments are tabulated in Appendix Table 3.9 together with data from other blots and the data are summarised diagrammatically in Figure 3.19.



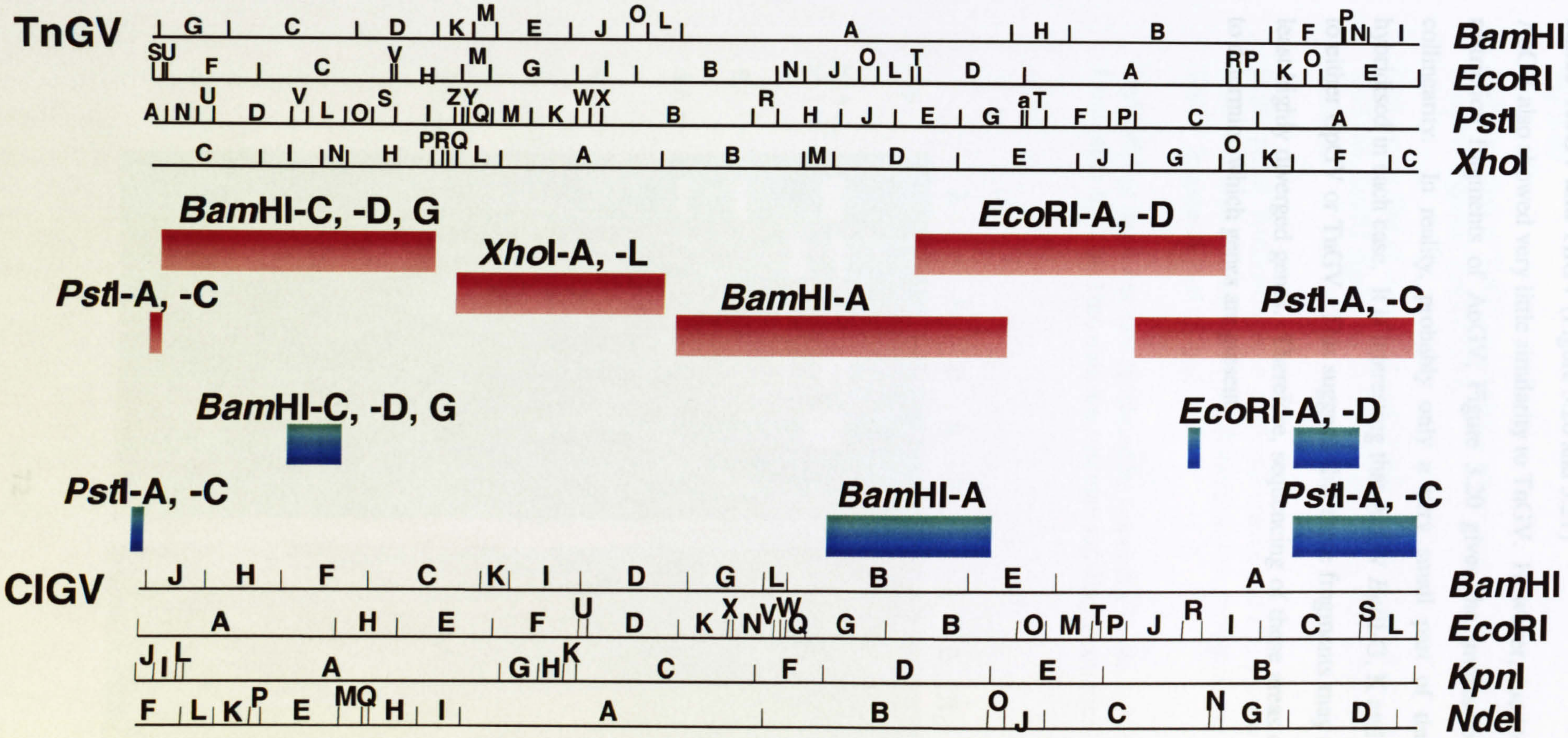


Figure 3.19
Collinearity of TnGV and ClGV genomes.
Physical maps of TnGV and ClGV are shown at the top and bottom respectively (Hashimoto *et al.*, 1996; Jehle *et al.*, 1992). Both genomes are drawn to the same length and are linearised at the granulin gene. TnGV fragments of DNA are indicated below the TnGV map (red boxes). Blue boxes above the ClGV map indicate regions of the ClGV genome that hybridised to TnGV fragments of DNA at low stringency (55°C).

3.2.10 AoGV and TnGV (Figure 3.20 and 3.21)

AoGV also showed very little similarity to TnGV. However, due to the large size of the restriction fragments of AoGV, Figure 3.20 gives the impression of similarity and collinearity. In reality, probably only a very small part of these large fragments hybridised in each case. It is interesting that AoGV *Bgl*II-G, K and -J did not hybridise to either CpGV or TnGV. This suggests that these fragments may contain unique or at least highly diverged genes. Therefore, sequencing of these areas could be undertaken to determine which genes are present.

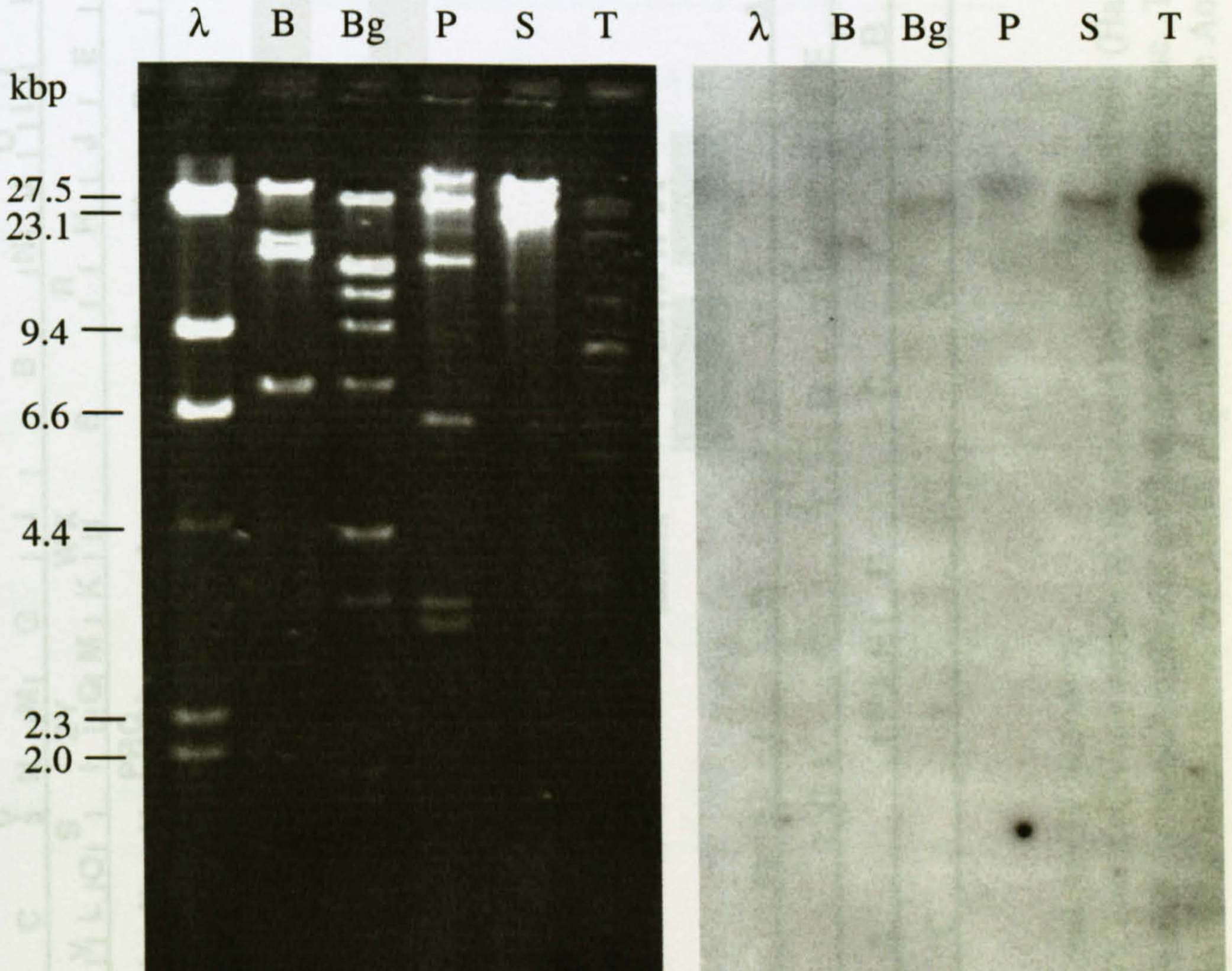
Fig 3.20

Hybridisation of TnGV fragments *Pst*I -A and -C to a blot containing restriction enzyme digested AoGV DNA

Left panel: Gel of digests of AoGV with *Bam*HI (B), *Bgl*II (Bg), *Pst*I (P) and *Sac*I (S). *Eco*RI digests of TnGV (T) and DNA size ladder λ *Hin*dIII (λ) are included.

Right panel: Autoradiograph of membrane-bound DNA from the same gel, after Southern blotting and hybridisation at low stringency to ^{32}P dCTP-labelled TnGV fragments *Pst*I -A and -C.

Hybridising fragment sare tabulated in Appendix Table 3.10 together with data from other blots and the data are summarised diagrammatically in Figure 3.21.



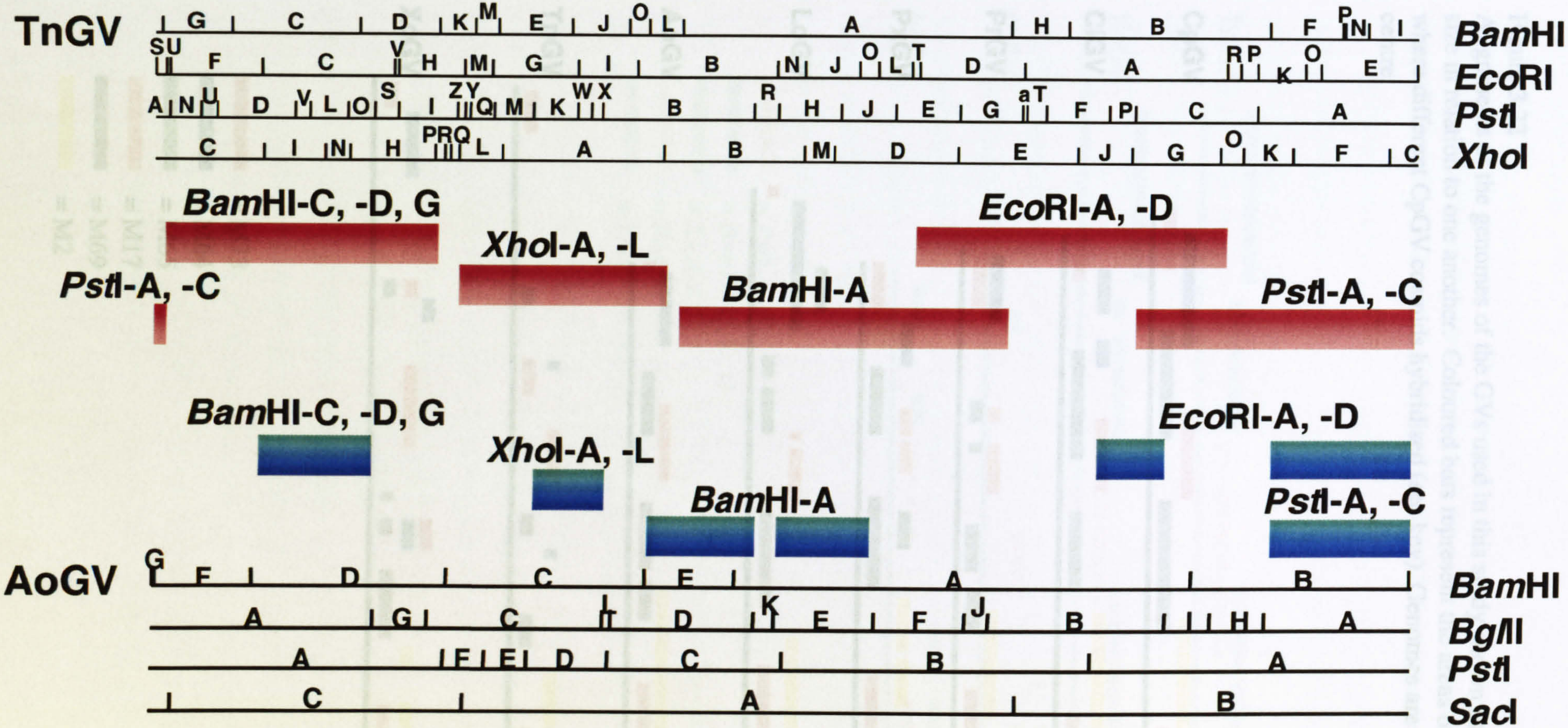


Figure 3.21
Collinearity of TnGV and AoGV genomes.
Physical maps of TnGV and AoGV are shown at the top and bottom respectively (Hashimoto *et al.*, 1996; Figure 4.16).
Both genomes are drawn to the same length and are linearised at the granulin gene. TnGV fragments of DNA are indicated below the TnGV map (red boxes). Blue boxes above the AoGV map indicate regions of the AoGV genome that hybridised to TnGV fragments of DNA at low stringency (55°C).

Figure 3.22
Alignments of the genomes of the GVs used in this study shown as actual genome size in relation to one another. Coloured bars represent the areas of the genome where different CpGV cosmids hybridised (see key). Genomes are aligned from their centre.

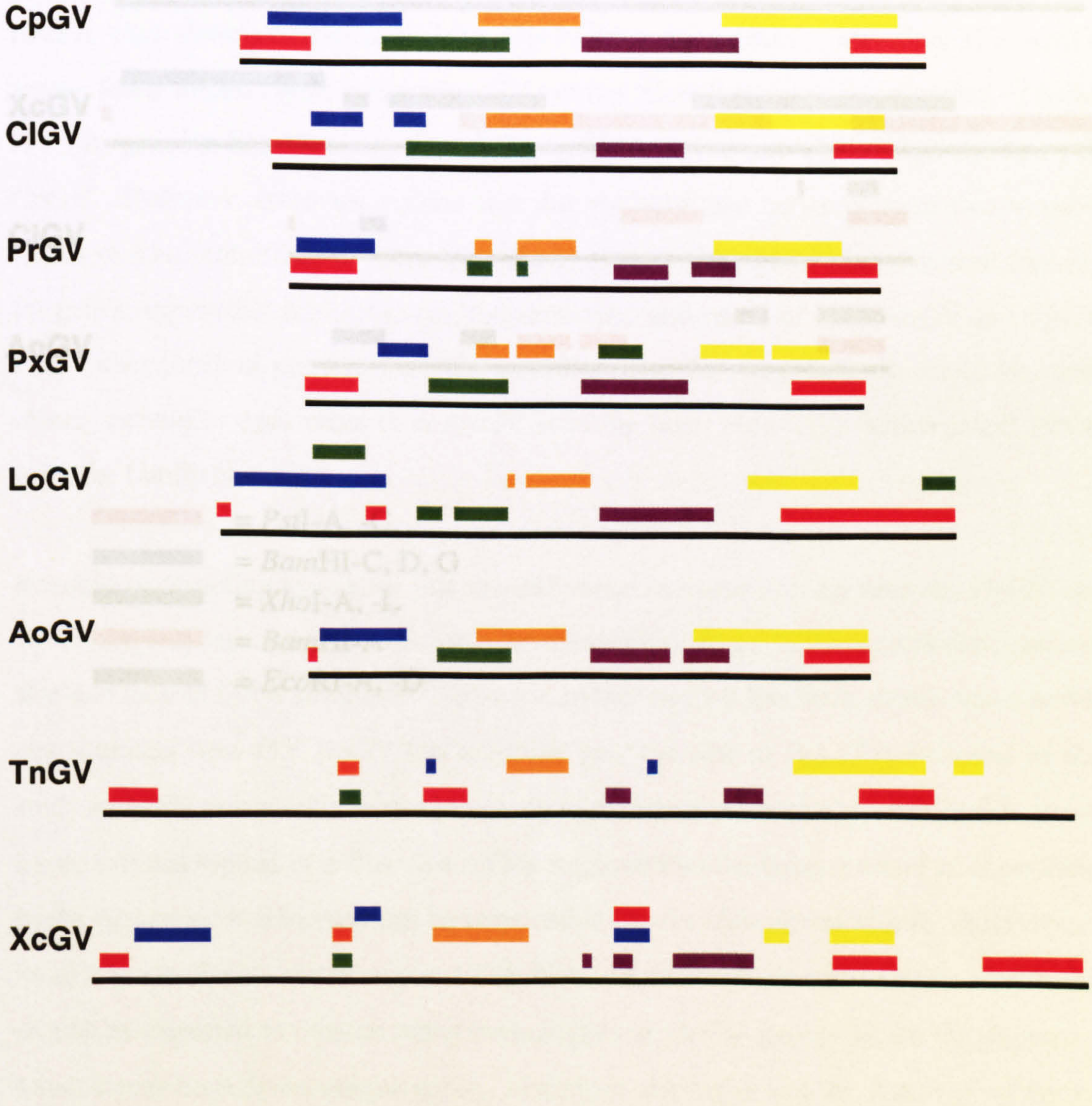
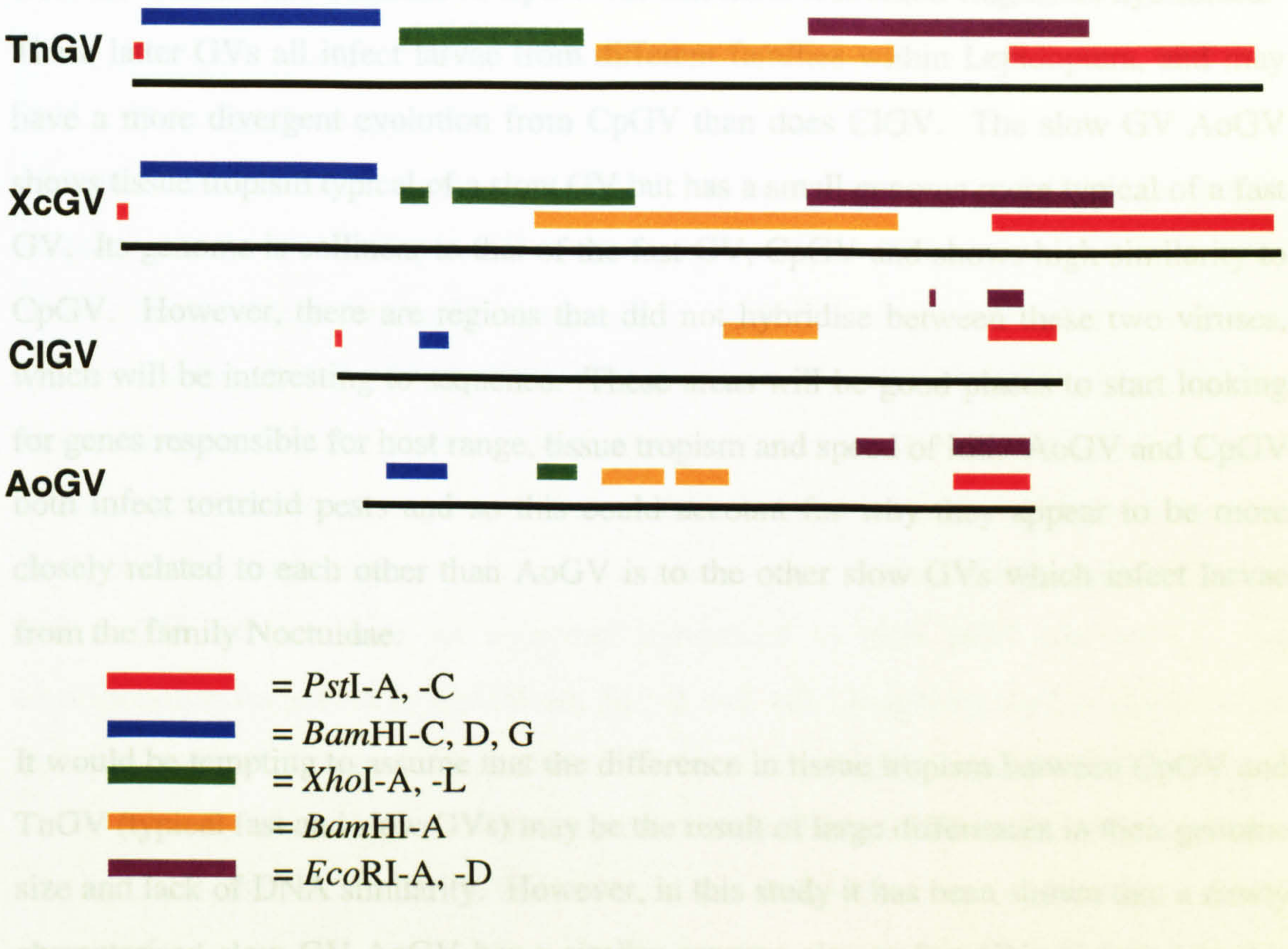


Figure 3.23
Alignments of the genomes of GVs shown as actual genome size in relation to one another. Coloured bars represent the areas of the genome where different TnGV fragments hybridised (see key). Genomes are aligned at their centre.



3.3 Discussion

From these studies a number of conclusions could be drawn. Firstly, the two slow GVs, TnGV and XcGV were highly similar and collinear to each other but showed very little similarity to fast GVs. Secondly, fast GVs displayed variable amounts of collinearity and similarity to each other. CpGV and ClGV show a high degree of similarity, to an extent comparable to that observed between XcGV and TnGV. The other fast GVs, were less similar and collinear to CpGV but still most restriction fragments hybridised. These latter GVs all infect larvae from different families within Lepidoptera, and may have a more divergent evolution from CpGV than does ClGV. The slow GV AoGV shows tissue tropism typical of a slow GV but has a small genome more typical of a fast GV. Its genome is collinear to that of the fast GV, CpGV and shows high similarity to CpGV. However, there are regions that did not hybridise between these two viruses, which will be interesting to sequence. These areas will be good places to start looking for genes responsible for host range, tissue tropism and speed of kill. AoGV and CpGV both infect tortricid pests and so this could account for why they appear to be more closely related to each other than AoGV is to the other slow GVs which infect larvae from the family Noctuidae.

It would be tempting to assume that the difference in tissue tropism between CpGV and TnGV (typical fast and slow GVs) may be the result of large differences in their genome size and lack of DNA similarity. However, in this study it has been shown that a newly characterised slow GV AoGV has a similar genome size to fast GVs included in this study and also shares with them a relatively high degree of similarity. Yet AoGV shows tissue tropism typical of a slow GV. This suggests that the large amount of extra DNA in the typical slow GVs may not be responsible for the slow speed of kill. AoGV has a relatively small genome of about 100.9 kbp and shows high similarity to CpGV and would be expected to contain many homologues of CpGV genes. Since the genome is small it may have fewer unique genes. Therefore, the regions of the AoGV genome that do not hybridise to CpGV could contain unique genes involved in its tissue tropism and speed of kill. Whether a similar gene or genes are responsible for the slow speed of kill of other GVs is impossible to predict but it is extremely worthy of investigation.

There are regions of the CpGV genome that rarely hybridised to other GV genomes. Firstly, for example, although the cosmids M17 and M69 contain adjacent sequences from the CpGV genome, there is a gap, sometimes quite large between the corresponding regions of hybridisation with the other GVs. This suggested that there are differences around this part of the genome. This region of CpGV contains a cluster of *Sal*I sites and is currently being sequenced and it will be interesting to look at the genes that are contained within this area. Secondly, all of the viruses studied, apart from LoGV and ClGV, lack a region in their genomes that can hybridise to both M64 and M65. These results suggest that this region may contain some unusual genes. The relevant DNA, the *Sal*I-L fragment of CpGV, has been sequenced and found to contain unique genes (D. Winstanley and D. O'Reilly, personal communication). Thirdly, none of the viruses, except for AoGV, contained a region in their genomes that was able to hybridise both to M69 and M2. This corresponded to *Sal*I-K of CpGV, which contains part of the gene *very late factor 1* (*vlf-1*) along with four small baculovirus homologues of unknown function. It is possible that AoGV also lacks this area of hybridisation as the hybridising fragments around that region were large. Lastly, the genomes of PxGV and AoGV did not have an area that hybridised to both M73 and M64. The corresponding fragments in CpGV are *Sal*I-S and –G. Fragment *Sal*I-G contains the cathepsin and chitinase genes, which are relatively highly conserved genes. As discussed earlier, homologues of these genes have not been found in the PxGV genome. This may also be the case for AoGV.

The results from this chapter suggest that GVs from insect hosts belonging to the same family appear to share greater genome similarity and collinearity than GVs from insect hosts of different families. Tissue tropism of a GV is not dependent on the taxonomy of the host. The key to finding genes responsible for tissue tropism may lie in looking at regions of the genome where significant differences as judged by lack of similarity, are observed.

CHAPTER 4

Biological and molecular studies of *Adoxophyes orana* granulovirus (AoGV), a slow killing GV

4.1 Introduction

Virus was isolated from overwintering caterpillars obtained from two orchards in Kent in 1993. It was found to be a mix of a granulovirus (GV) and a nucleopolyhedrovirus (NPV). The granulovirus genome has been compared to other GVs (chapter 3) and was found to show most similarity to those of fast GVs with wide tissue tropism. However, biologically the virus appeared to be more similar to slow GVs, which have a narrower tissue tropism, infecting only the fat body (Federici, 1997).

Prior to this study, AoGV had not yet been fully characterised, either by detailed bioassay or molecular methods. However, preliminary restriction enzyme studies have been performed on the genome of a Swiss strain of AoGV (Drolet, 1989). The genome of the English isolate is relatively small for a GV at approximately 100.9 kbp. This chapter contains preliminary studies on the pathogenicity and genome of this virus. Detailed dosage-mortality and time-mortality studies were performed on various instars. These studies also allowed careful observations on the gross pathology of the virus infection.

As a first step towards a more detailed characterisation of the genome of the English AoGV isolate and its relation to other GVs, a physical map was constructed. The genome could then be compared to other GVs by hybridisation (chapter 3). The mapping also aided in the sequencing of the granulin containing area. This was a primary step towards the complete sequencing and analysis of its genome. The gene organisation of this area was then compared to other GVs.

4.2 Results

4.2.1 Determination of the larval instars and development time of *Adoxophyes orana*

The head capsule sizes of ten *A. orana* neonate larvae, which had hatched within a 0-6 hour period, were measured daily using a graduated eyepiece in a light microscope. The larvae were measured at the widest part of their head capsule. From these results an average head capsule size for each instar could be calculated, Table 4.1. Once the larval instars had been determined, a study was undertaken to determine the development cycle of healthy *A. orana* larvae. This was a prerequisite to performing bioassays, which would require an accurate knowledge of the larval instars.

Seventy-five neonate larvae which had hatched within a 0-6 hour period were placed in individual wells of a 25 well plate containing a piece of semi-synthetic artificial diet containing formalin (Appendix 1). These larvae were observed daily from hatching to determine their larval instar until pupation and finally emergence as moths, this is depicted in Figure 4.1. The developmental time (DT_{50}) was interpolated from the graph and is shown in Table 4.2.

Table 4.1 Average size and standard deviation of the head capsule of *A. orana* in each larval instar (n = 10).

Larval instar	Head capsule width (mm)
1	0.23 ± 0.012
2	0.33 ± 0.005
3	0.48 ± 0.019
4	0.79 ± 0.055
5	1.14 ± 0.067

Figure 4.1

Development of healthy *Adoxophyes orana* larvae at 25°C. P = Pupae, A = Adults, 1-5 = larval instars. Seventy-five *A. orana* larvae were observed daily from hatching to determine their larval instar.

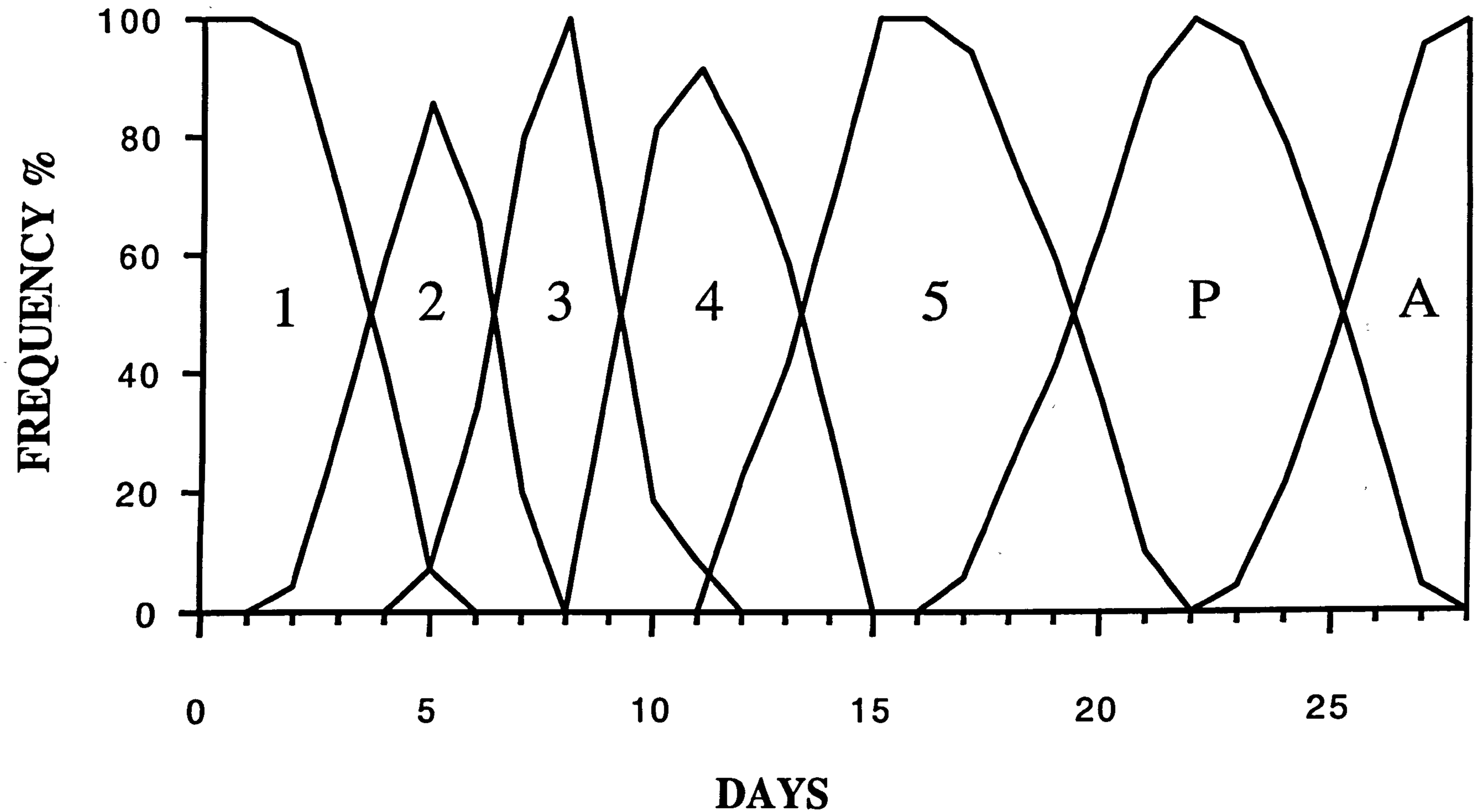


Table 4.2 Developmental time in days, of healthy *A. orana* at 25°C from hatching until 50% of the larvae reached the given instar (DT_{50}).

Larval Instar	DT_{50} (days)
2	3.7
3	6.3
4	9.2
5	13.3
Pupae	19.4
Adults	25.2

4.2.2 Purification of AoGV from AoNPV

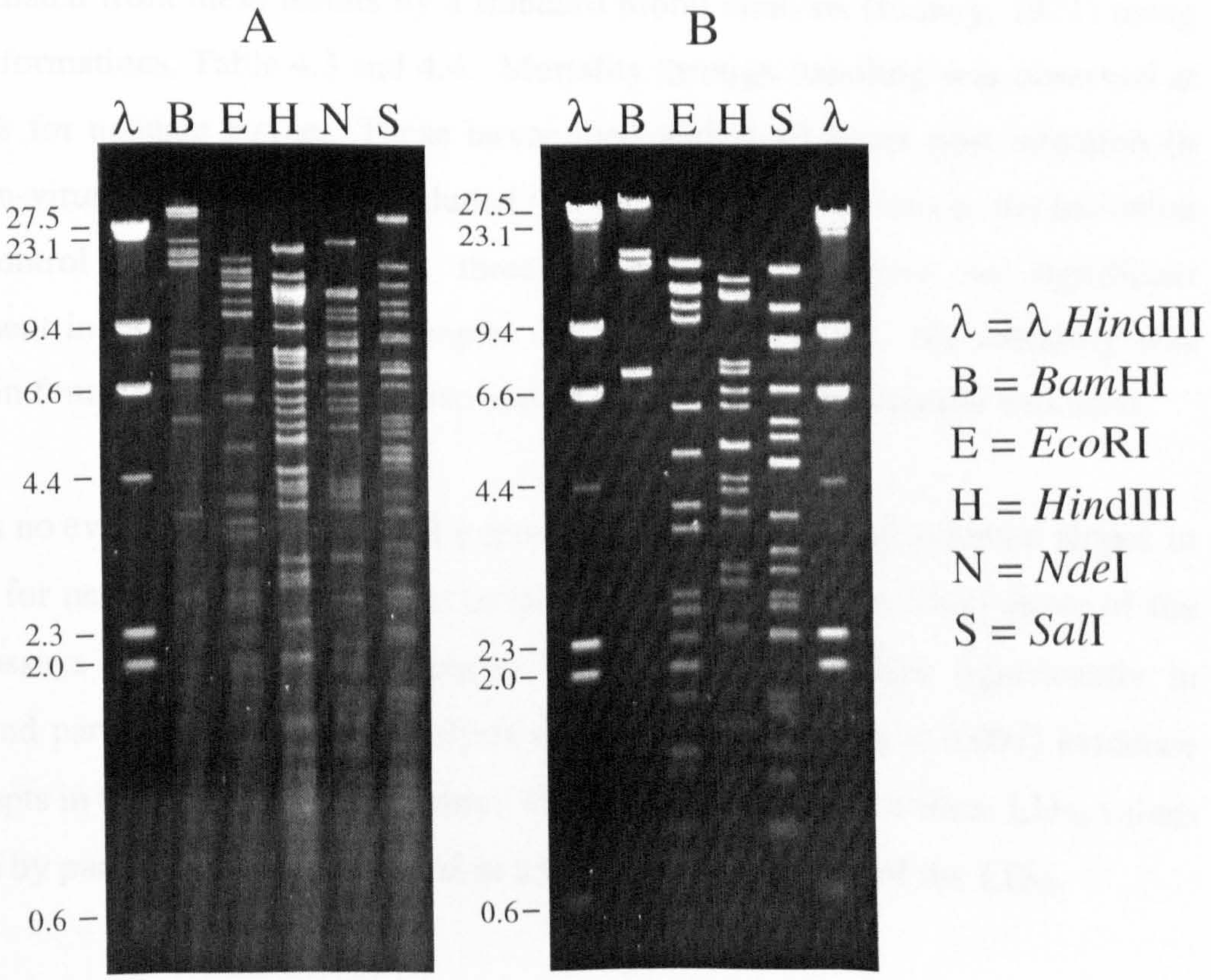
The virus-infected larvae collected in 1993 had been frozen at –20°C for 5 years. The larvae were very small so virus from these was crudely purified and fed to fourth instar larvae to increase the virus yield. The larvae died between 7 and 23 days post infection (d p.i.). Virus was again crudely purified and the virus DNA extracted (sections 2.7.1.3 and 2.7.4). The DNA restriction endonuclease profiles indicated that one virus was present due to the lack of submolar bands. However, when the profile was compared to that of the Swiss strain of AoGV (AoGV-S) (Drolet, 1989) it was found that there were a lot fewer bands in AoGV-S, all of which were present in the English virus profile. This suggested that the English virus contained an equal mix of two viruses, Figure 4.2 A.

The virus was viewed under the light microscope and appeared to be about 90% GV and 10% NPV. Although there was more GV than NPV present, each NPV OB is likely to contain more DNA than a GV OB due to multiple virions embedded within the polyhedrin which may account for the equal amount of DNA of the two viruses. The virus was then purified further on glycerol and sucrose gradients to separate the GV and NPV components (section 2.7.1.1). The GV profile obtained from this purified virus appeared clean with no contaminating bands and was the same as that published for the Swiss isolate of AoGV (Drolet, 1989), Figure 4.2 B.

The GV was checked under EM for the presence of NPV and none were found. The virus was passed three times in fourth instar larvae at an LD_5-LD_{20} dose to verify the elimination of NPV. Therefore, this virus was considered pure GV and named AoGV-E.

The virus was counted using a counting chamber (section 2.7.3) and stored at –20°C in 200 µl aliquots at a concentration of 1 x 10^{10} OBs/ml. This stock was the reference source used for the bioassays.

Figure 4.2. Restriction digests of A) DNA extracted from the original English *A. orana* virus mix and B) purified English AoGV from the virus mix (same profile as Swiss AoGV), run on a 0.7% agarose gel.



4.2.3 Diet based bioassays of AoGV-E

All bioassays were performed at 25°C using neonates, fourth and fifth instar *A. orana* larvae. The volume of liquid ingested by neonates 0-6 hours post-hatch was calculated to be 5.35 nl ± 0.945 nl using a fluorescence spectroscopy method developed by van Beek and Hughes (1986) (section 2.6.2). To determine the dosage-mortality responses, neonates were fed with appropriately diluted virus using the micro-droplet feeding procedure (Hughes and Wood, 1981; Hughes *et al.*, 1986) (section 2.6.3). Fourth and fifth instar larvae were infected with AoGV-E within 24 hours post-moult (section 2.6.4). Larvae were checked once a day for deaths and symptoms were noted.

Fifty larvae were dosed at each of five virus dilutions. Each bioassay was performed three times. The raw data are shown in Appendix 4. LD_{50}, LD_{80} and LD_{95} values were calculated from these results by a standard probit analysis (Finney, 1971) using logit transformations, Table 4.3 and 4.4. Mortality through handling was observed at about 14% for neonate larvae. These larvae died within 48 hours post infection (h p.i.) of non-virus deaths and were excluded from the final calculations as the inclusion of the control parameter using a three-parameter model gave no significant improvement in fit relative to the simpler two-parameter model. No mortality was observed in fourth and fifth instar larvae and so the two-parameter model was used.

There was no evidence for differential intercepts (parallelism) or differential slopes in bioassays for neonates and fifth instar larvae. Therefore, a single fitted curve of the three bioassays was used, as the separate bioassays did not differ significantly in position and parallelism. Parallel analysis showed very strong ($p < 0.001$) evidence for intercepts in the fourth instar bioassay. Therefore, a mean of the three LD_{50} values calculated by parallel analysis was used as an alternative estimate of the LD_{50}.

Table 4.3 LD_{50} values of AoGV-E for neonate (1), fourth and fifth instar larvae of *A. orana*. The fourth instar LD_{50} values for each bioassay calculated by parallel analysis are shown along with fiducial limits, as fiducial limits for an average of the three could not be calculated.

Instar of larvae	LD_{50} (OBs)	Lower fiducial limit	Upper fiducial limit	Slope	SE of slope	χ^2	Degrees of freedom
1	30.5	25.6	36.1	2.84	0.214	14.25	13
4 (1)	2.83×10^3	1.71×10^3	4.59×10^3				
4 (2)	1.45×10^4	9.01×10^3	2.45×10^4				
4 (3)	5.40×10^3	3.40×10^3	8.73×10^3				
4 (mean)	6.00×10^3			1.71	0.132	15.21	11
5	1.36×10^6	5.41×10^5	1.94×10^6	1.17	0.088	17.68	13

Table 4.4

Approximate LD_{50}, LD_{80} and LD_{95} values for *A. orana* larvae infected with AoGV-E at different instars.

Larval Instar	**LD_{50} (OBs)**	**LD_{80} (OBs)**	**LD_{95} (OBs)**
1	30.5	93.6	331
4	6.00×10^3	4.86×10^4	3.19×10^5
5	1.36×10^6	2.08×10^7	4.45×10^8

4.2.4 Calculation of median survival time (ST_{50})

LD_{80} doses were fed to groups of 75 larvae in triplicate to calculate the median survival time (ST_{50}). The ST_{50} refers to the time required for 50 % of the larvae to die after receiving a dose of virus. The experimental set up was such that all of the larvae received a particular dose within a short time span and were then held on non-contaminated diet throughout the remaining time of the test (Hughes *et al.*, 1997). The raw data are shown in Appendix 4.

ST_{50} calculations were based on the total number of larvae responding to virus infection, rather than the total number of larvae used in each test (Allaway and Payne, 1984).

For each instar the point in time at which 50% of the total mortality response occurred was interpolated from the sigmoid response curve, Table 4.5. The mean ST_{50} was calculated from the three assays for each genotype. The mortality curves for the averages of the three replicate bioassays performed on each developmental stage are shown in Figure 4.3.

Table 4.5

Mean ST_{50} values of the three bioassays for each instar and the standard error of the mean (s.e.) for *A. orana* larvae infected with an LD_{80} dose of AoGV-E at different instars.

Larval instar	ST_{50} (days)	s.e. (days)
1	37.0	± 1.18
4	28.8	± 0.61
5	23.9	± 0.81

4.2.5 Observed signs of AoGV-E infection

The larvae were observed during the bioassays and the symptoms of infection were noted. Photographs of larvae infected as neonates, fourth and fifth instars are shown in Figure 4.4, 4.5 and 4.6, respectively. Regardless of which instar they were infected no obvious symptoms were observed until the larvae had reached fifth instar. The first difference noticed between the controls and the infected larvae, regardless of which instar the larvae were infected, was that unlike infected larvae, the controls had pupated within 4-6 days of reaching fifth instar. One to two days after the controls had pupated, whitening under the skin could be observed in the infected larvae. This was the fat body, which was beginning to whiten due to virus occlusion body

Figure 4.3
Mortality curves of the averages of the bioassays performed on neonate, fourth instar and fifth instar *Adoxophyes orana* larvae that had been infected with an LD_{80} dose of AoGV-E1 based on the total number of larvae responding to virus infection.

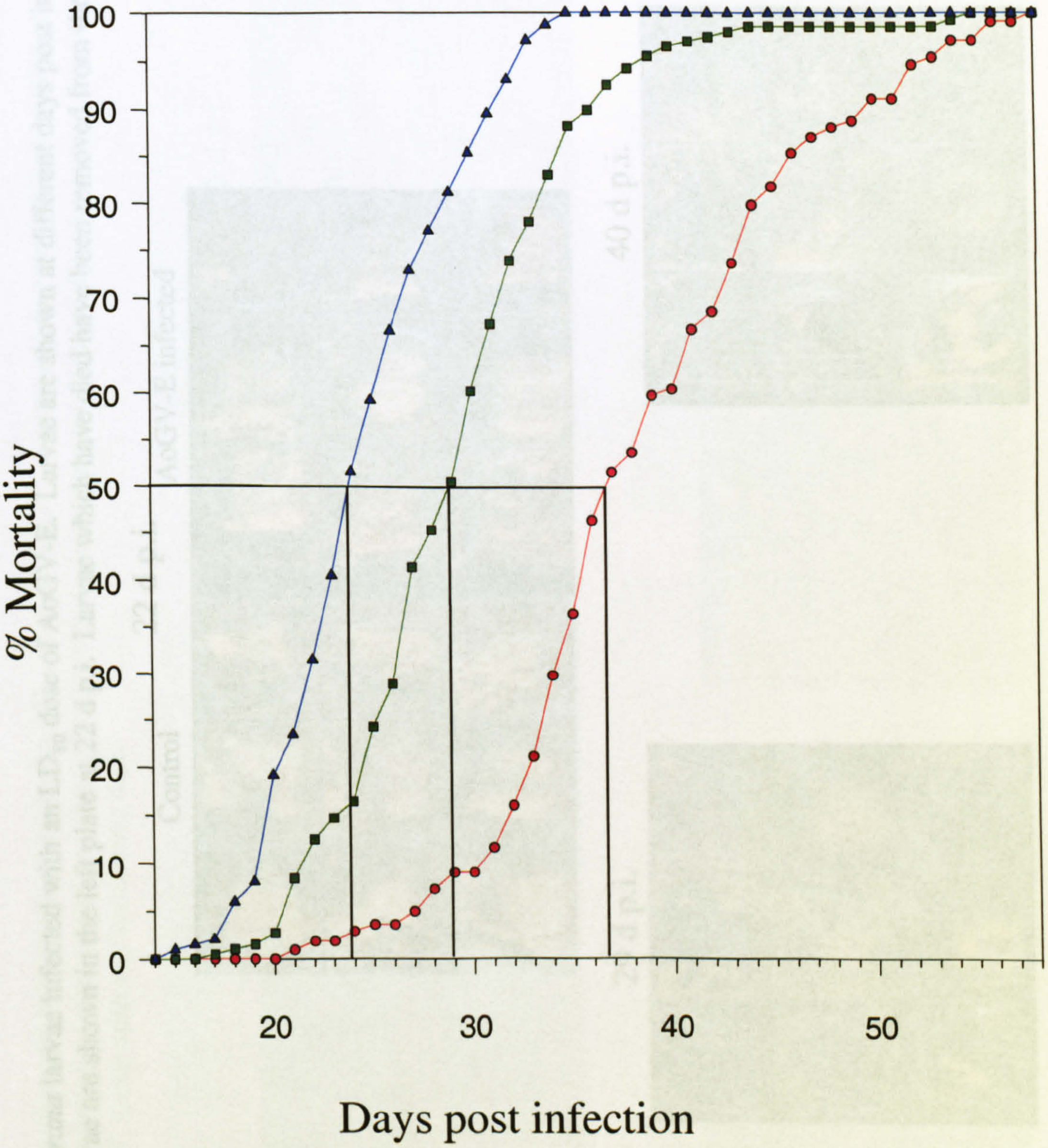
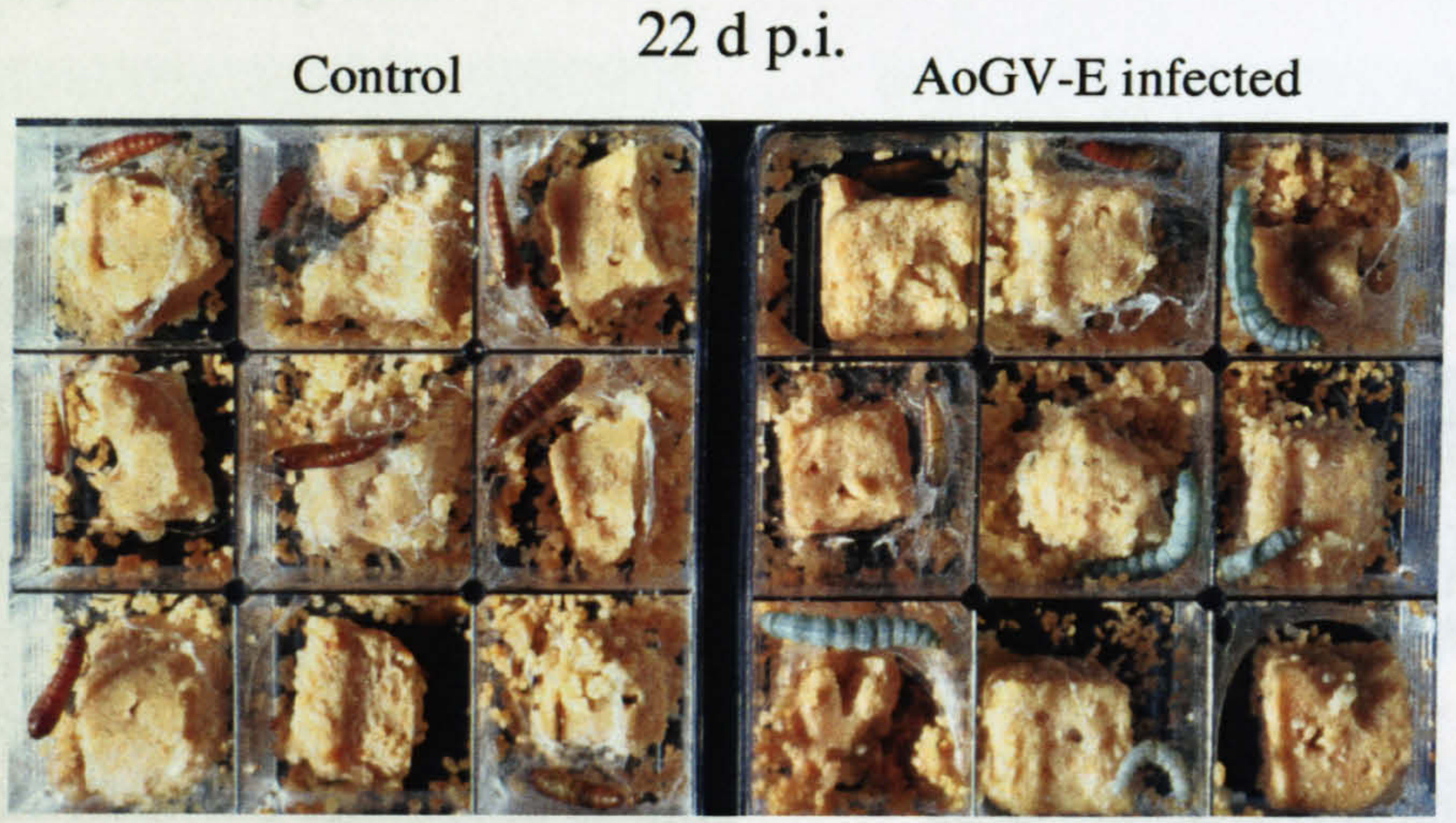
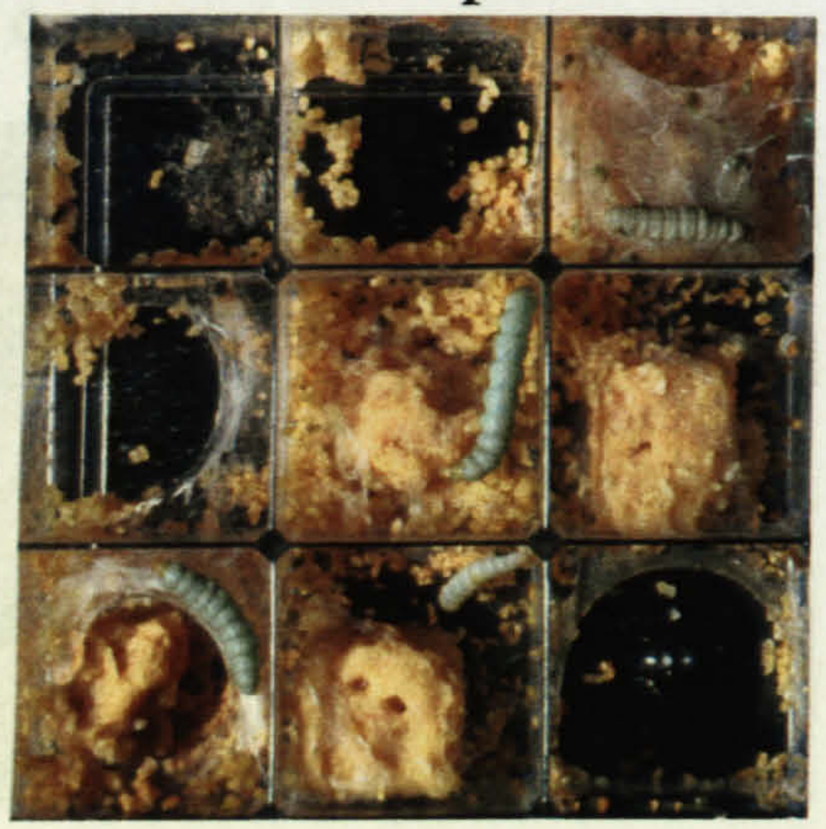


Figure 4.4
Neonate *Adoxophyes orana* larvae infected with an LD_{80} dose of AoGV-E. Larvae are shown at different days post infection (d p.i.). Uninfected control larvae are shown in the left plate at 22 d p.i. Larvae which have died have been removed from the well, along with any remaining diet.





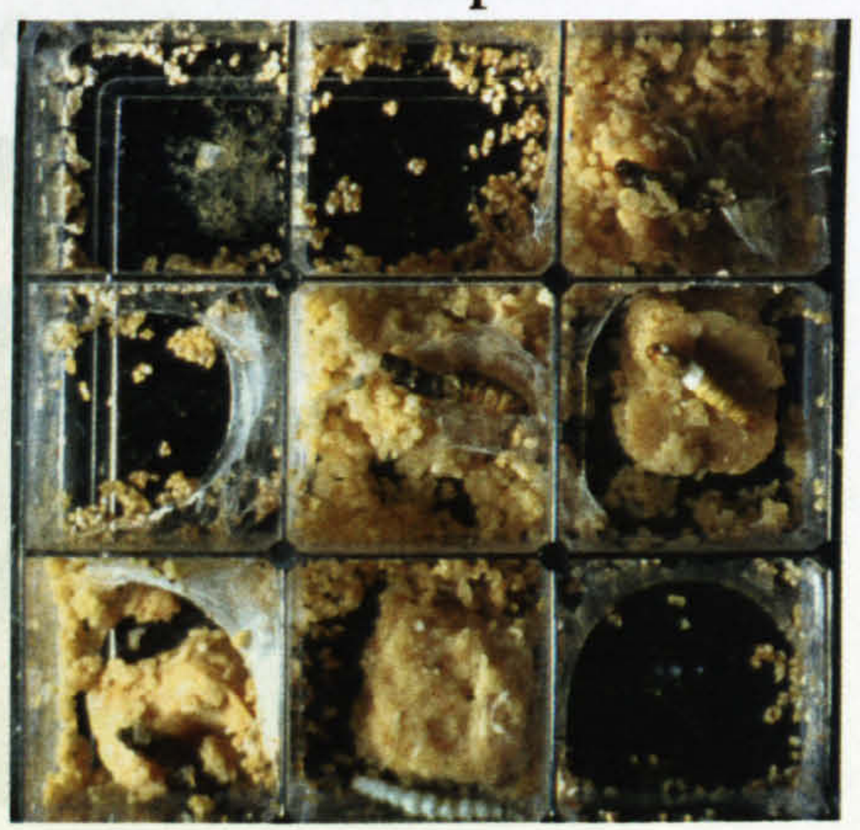
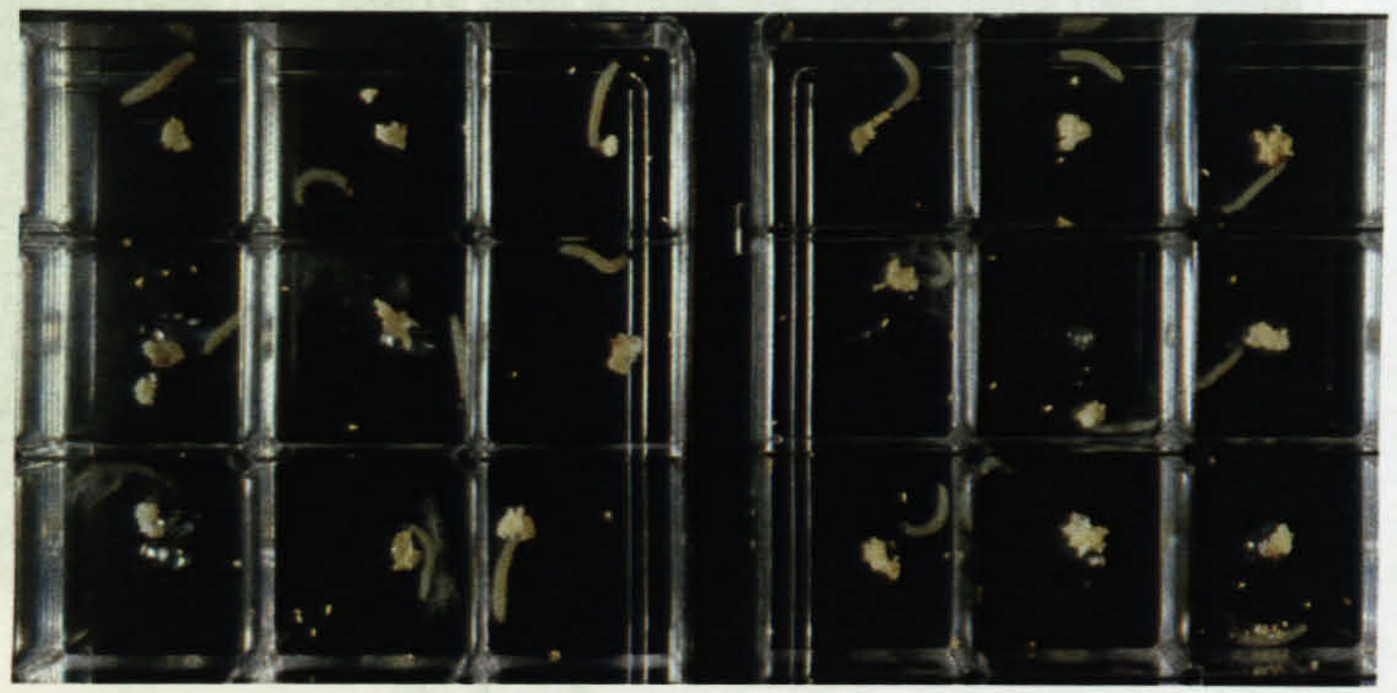
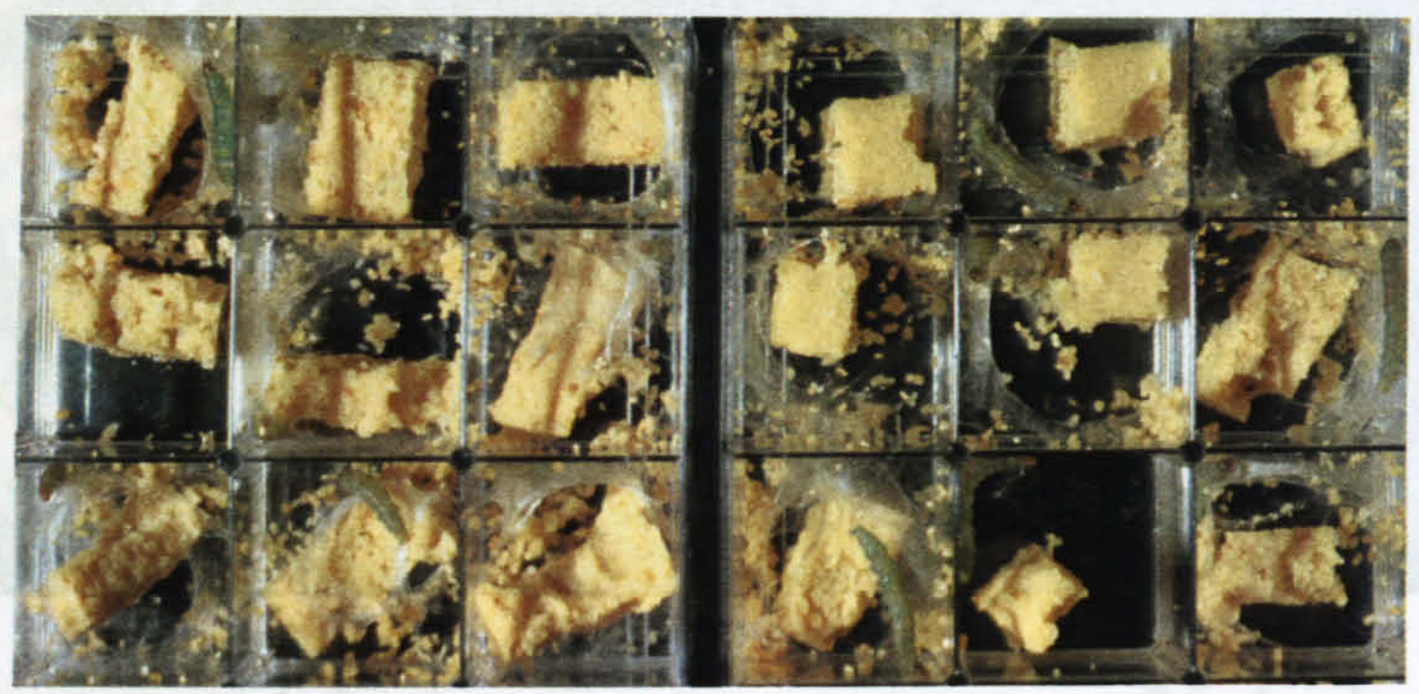
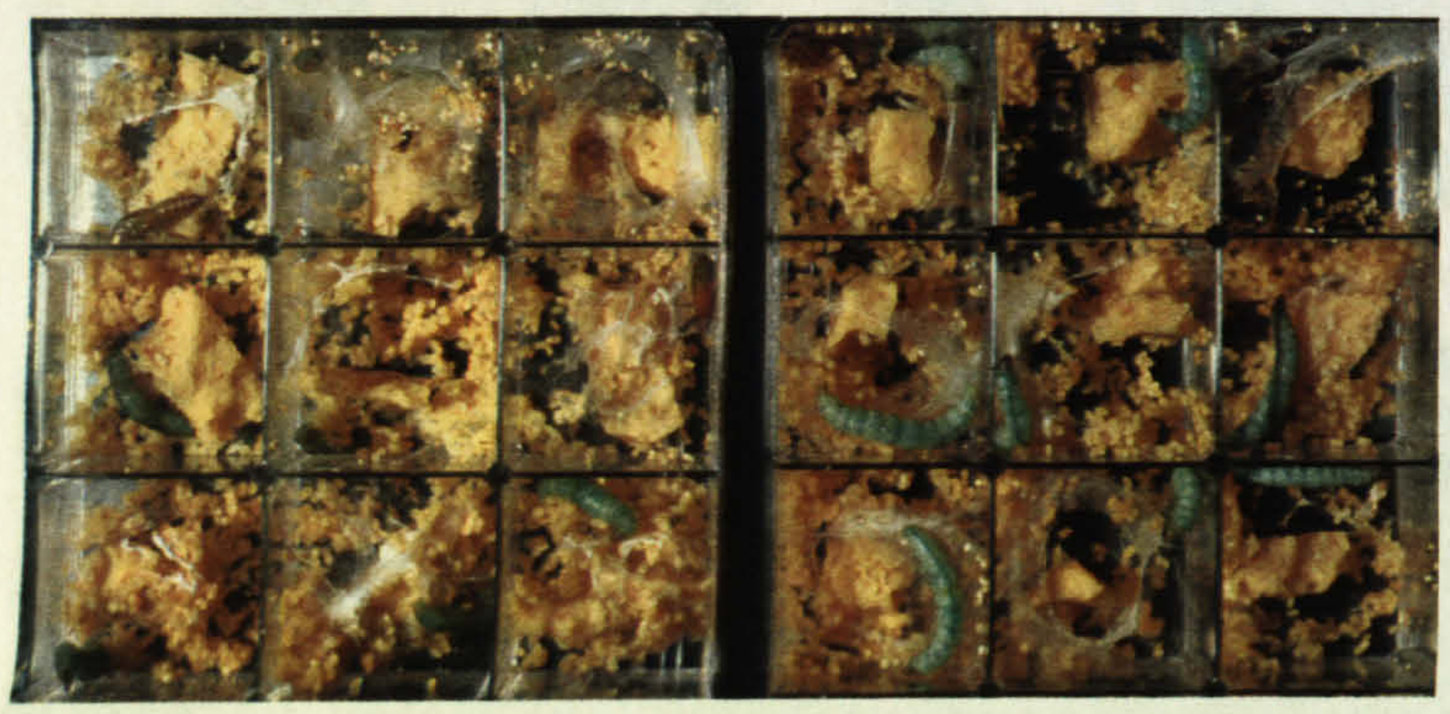
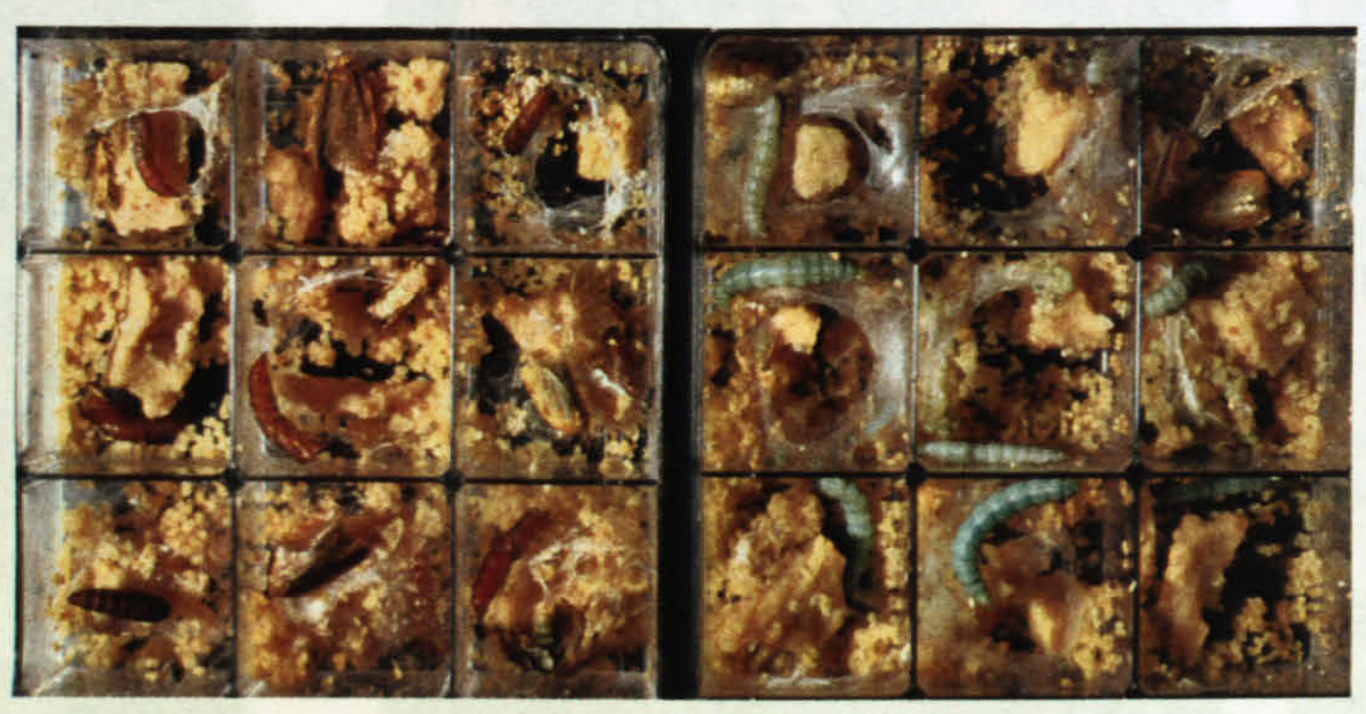


Figure 4.5
Fourth instar *Adoxophyes orana* larvae infected with an LD_{80} dose of AoGV. Larvae are shown at different days post infection (d p.i.). The left plates are uninfected control larvae and the right plates and single plate are infected larvae. Larvae which have died have been removed from the well, along with any remaining diet.









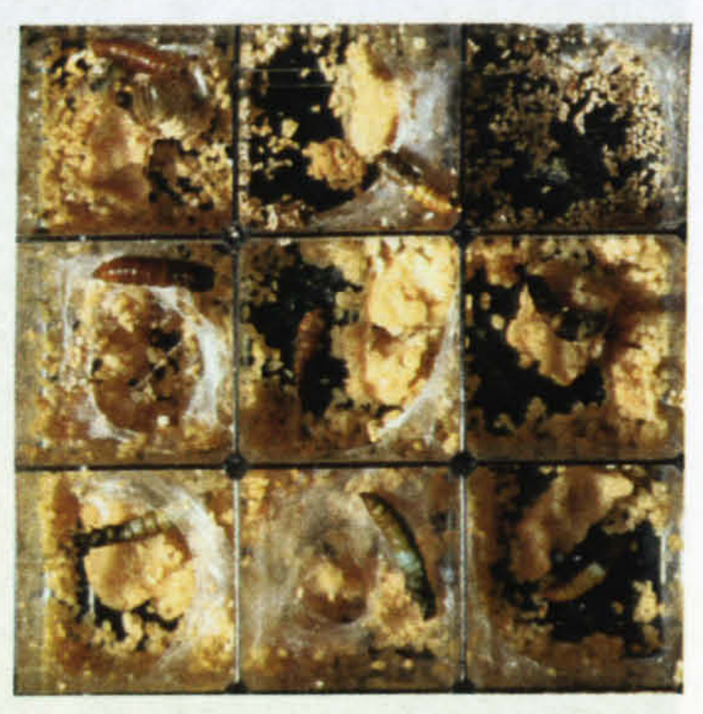
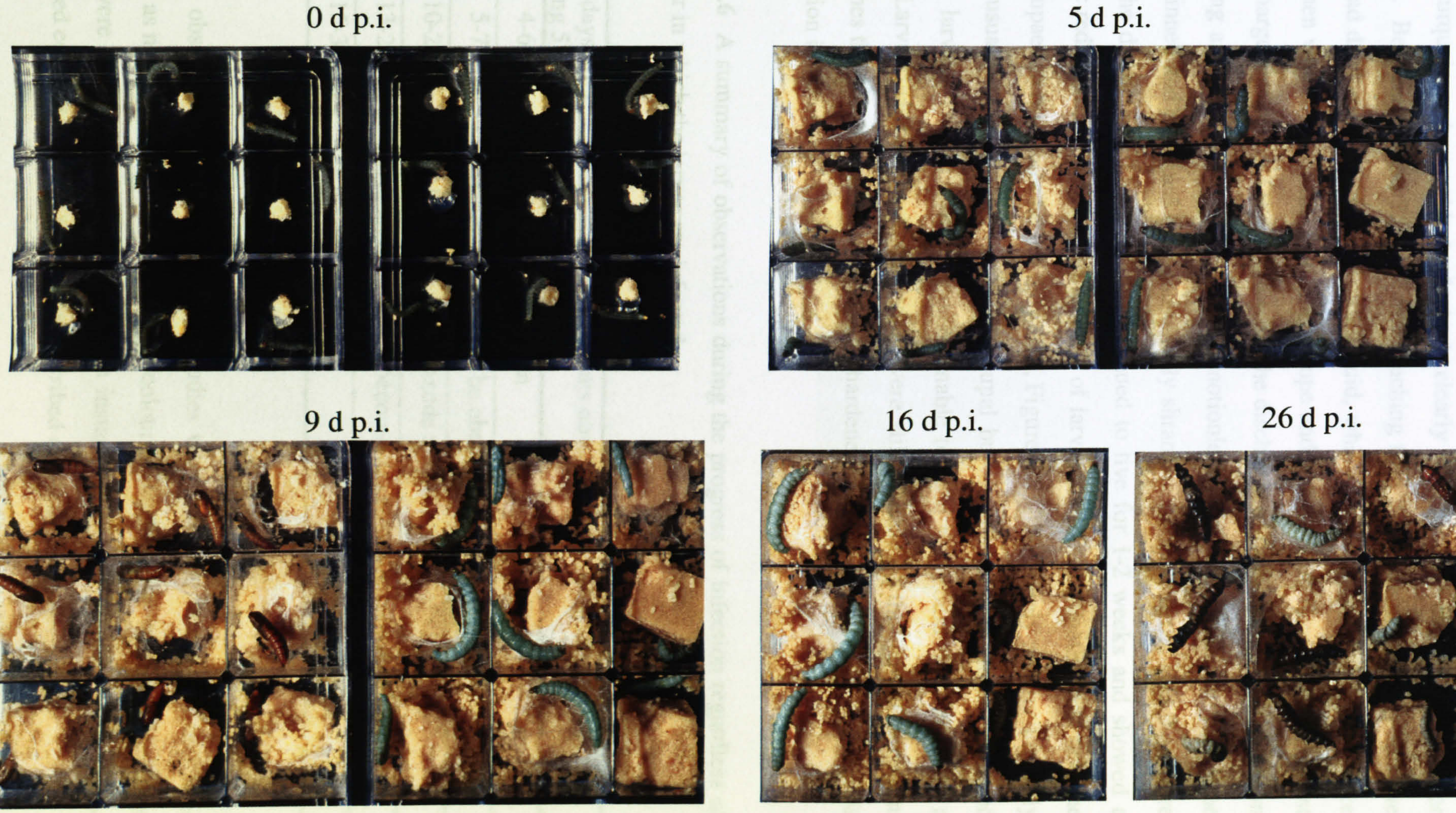


Figure 4.6
Fifth instar *Adoxophyes orana* larvae infected with an LD_{80} dose of AoGV-E. Larvae are shown at different days post infection (d p.i.). The left plates are uninfected control larvae and the right plates or single plates are infected larvae.



production. The larvae continued to feed and became whiter before turning into an overall turquoise colour, with the fat body clearly visible, white and enlarged, under the skin. Between 10 and 25 days after reaching fifth instar, approximately half the larvae had discharged a stream of milky fluid, which was a turquoise colour, Figure 4.7. When viewed under the light microscope under dark field, it could be seen that the discharge was full of OBs. Following the discharge of fluid there was a cessation of feeding and the larvae remained quite motionless unless agitated. They became loose-skinned and appeared grey and usually shrank to about half the size they were before the discharge. These larvae continued to live for 1-2 weeks and showed a dramatic reduction in feeding. About 85% of larvae attempted pupation and became larvae-pupae intermediates before they died, Figure 4.8. They rarely managed to fully pupate, usually their abdomen appeared pupal but their head and thorax appeared partially larval. These caterpillars were unable to feed but would live for about a week. Larvae that had discharged virus were still capable of this partial pupation. Sometimes the grey larvae just produced a hardened skin before dying. The progress of infection is summarised in Table 4.6.

Table 4.6 A summary of observations during the progress of infection regardless of the instar in which the larvae are infected.

No. days after reaching 5th instar	Symptoms observed in infected larvae
4-6	Absence of pupation
5-7	White fat body can be observed through the epidermis.
10-25	About 50% larvae exude a large stream of virus.
13-28	About 85% larvae become larvae-pupae intermediates.
15-35	Larvae die.

Another observation made during these studies was that some larvae that had been infected as neonates looked a white/yellow colour in fourth instar, unlike the controls, which were green. However, once in fifth instar these larvae looked similar to the uninfected controls until the symptoms described above developed.

Figure 4.7

A fourth instar larva infected with an LD_{80} dose of AoGV-E. A large volume of virus discharge has been emitted at 11 d p.i.

Figure 4.8

Fourth instar larvae infected with an LD_{80} dose of AoGV-E. The larvae exhibit a variety of larval/pupal intermediates before death. Larva A has actually emerged as a deformed moth.

Larva A

4.2.6 Time lapse imaging of virus-infected larvae

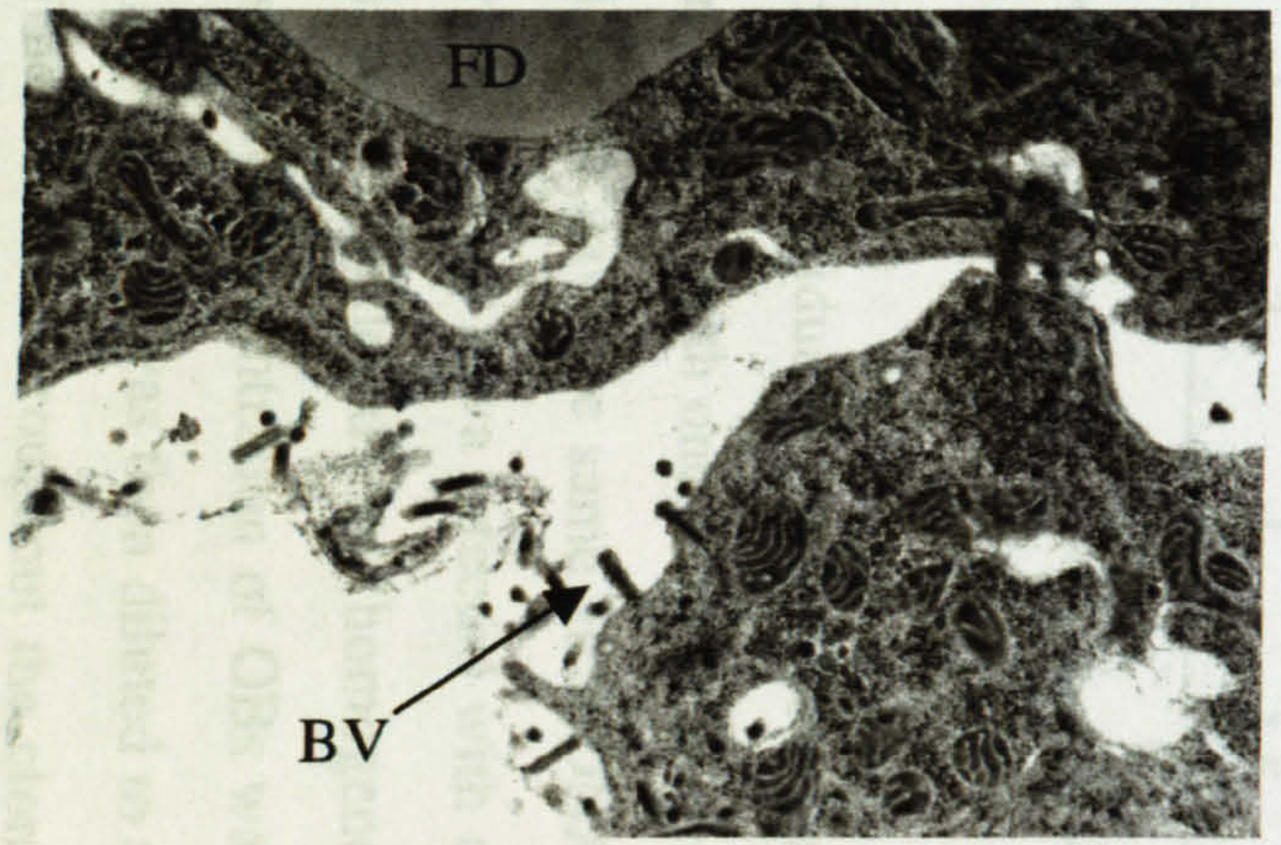
A time course video experiment was set up to determine whether the viral exudate was discharged from the anterior or posterior end of the larvae, as the moment of discharge had not been observed. The video camera was set up to overlook a 25 well plate of fourth instar larvae infected with an LD_{95} dose of AoGV, as calculated from the bioassays. The camera took a frame every 0.5 seconds. Therefore, a few days of infected larvae could be watched within a few hours. Two larvae were seen to discharge virus from their posterior suggesting it was eliminated from the gut. After discharge of the exudate, one of the larvae was observed to immediately start to ingest it. One other larva escaped from one well to another, consequently puncturing its epidermis and emitting the virus exudate. Both the uninfected and infected larvae were observed to possess a fragile epidermis. About half the larvae emitted the virus exudate and were small and grey but they were not actually seen emitting the virus. An observation made whilst examining larvae which were known to have emitted the exudate, was that on many there was a black spot about 0.5-1.0 mm near their rear end. This spot could be melanisation of a puncture wound, possibly the site of virus discharge. However, it was always in the same place on one or other side of the larvae. Therefore, the virus appeared to be emitted from the posterior end, either from the gut or from the haemocoel through a small puncture in the fragile epidermis.

4.2.7 EM studies of AoGV

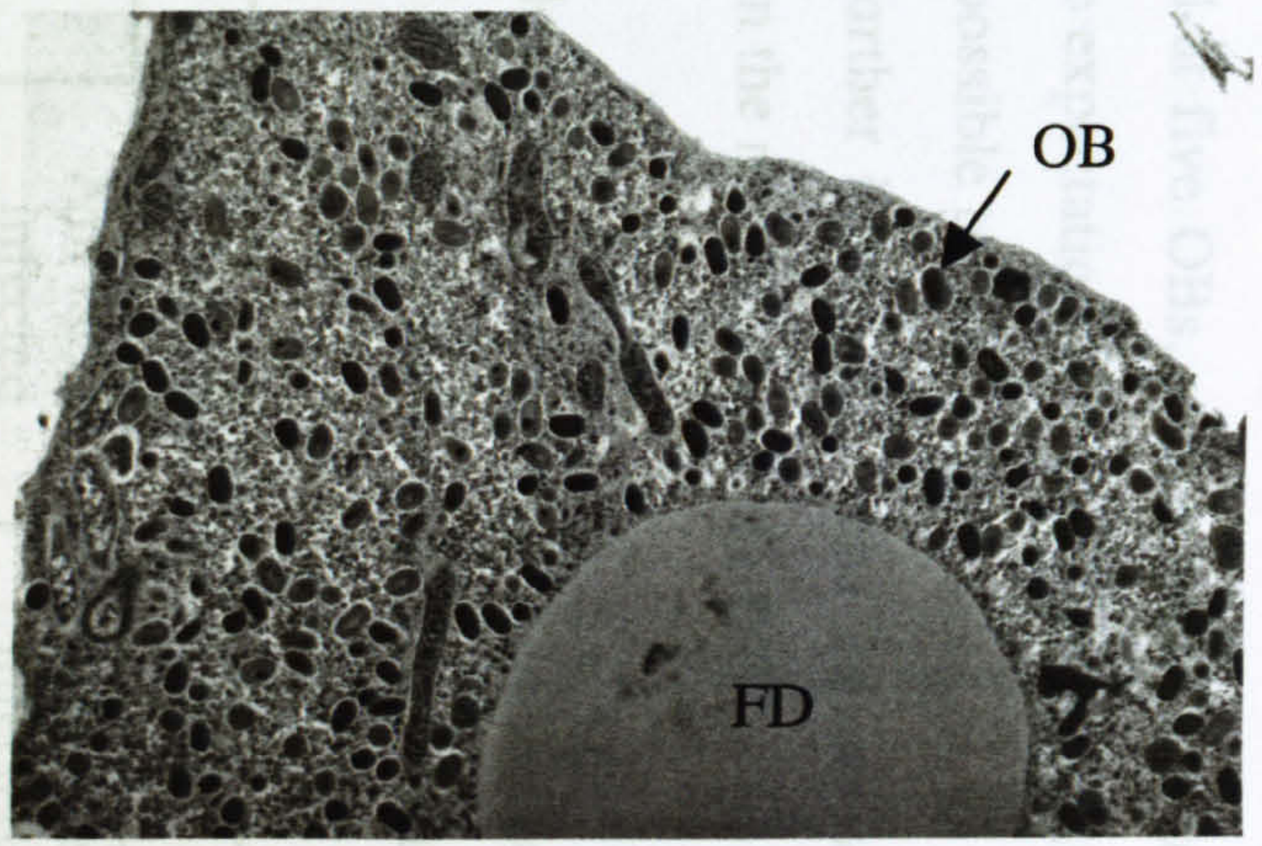
Fourth instar larvae were infected with an LD_{95} dose as calculated from the bioassays. Two larvae were taken at each time point, 4.5 d p.i., 7.5 d p.i., 10.5 d p.i. and 13.5 d p.i. These larvae were dissected and the midgut, floating fat body and segmental fat body fixed and embedded (section 2.11). Ultrathin sections were then viewed on the electron microscope for presence or absence of AoGV. Some cells of the floating fat body were found to be highly infected as early as 4.5 d p.i., Figure 4.9. The cells were filled with OBs. However, the cells were still intact and the organelles looked healthy. OBs were observed in the floating and segmental fat bodies of larvae at all time points. Budded virus was also observed exiting a fat body cell, Figure 4.9. OBs were not readily observed in the midgut although there seemed to be evidence of OBs in the lumen of the midgut late in infection. Abnormal aggregates of OBs were observed quite frequently in the fat body cells, Figure 4.9.

Figure 4.9
Electron micrographs of the fat body of AoGV-E infected *Adoxophyes orana* larvae.
1 = Budded virus entering/exiting a cell (4.5 d p.i.). 2 = An intact fat body cell filled with OBs (4.5 d p.i.). 3 = Abnormal aggregate of OBs within a proteinaceous matrix (10.5 d p.i.).

1

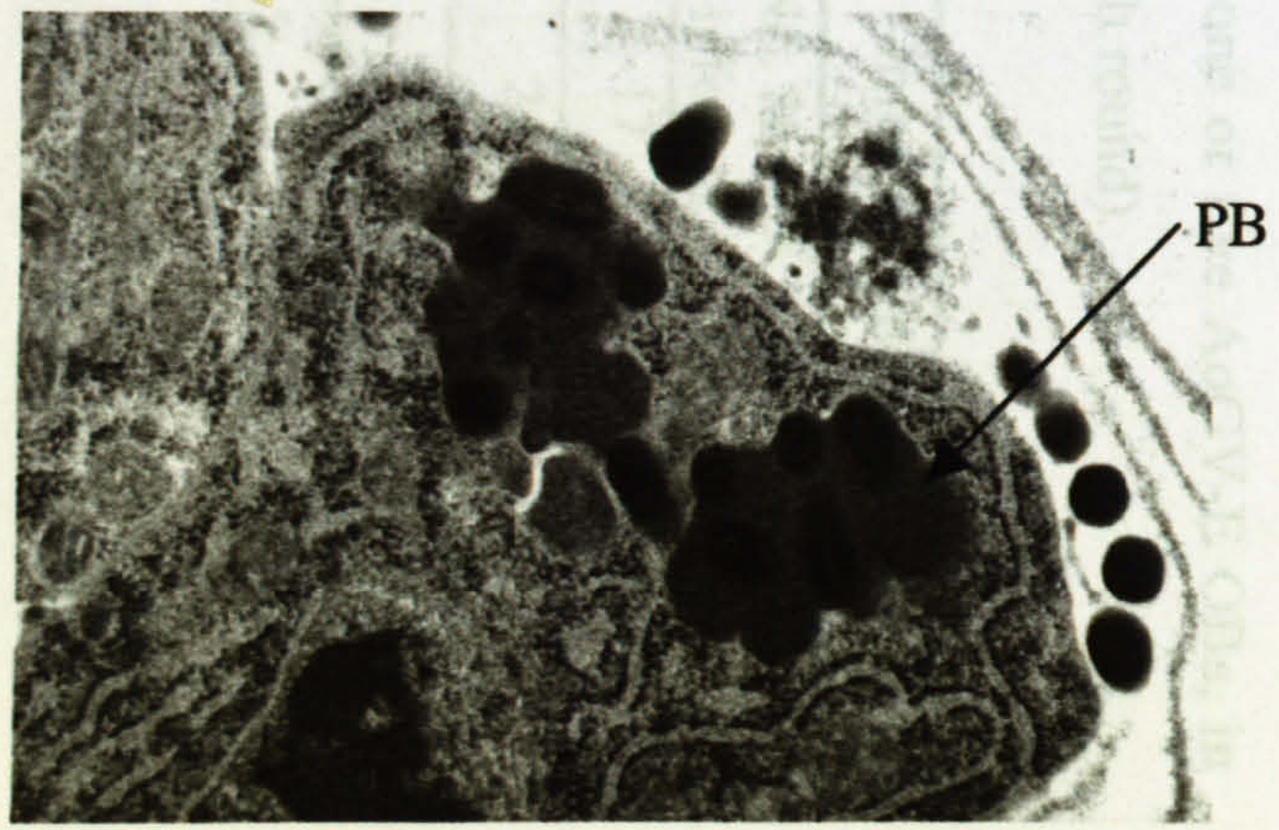


2



FD = Fat droplet
BV = Budded virion
OB = Occlusion body
PB = Proteinaceous matrix

3



4.2.8 *In vivo* cloning of AoGV-E

Although the virus had been crudely cloned, by the consecutive selections at LD_5-LD_{20}, and appeared homogenous by restriction endonuclease analysis, it was necessary to further clone the virus before any further work such as sequencing was performed on the virus that would require a cloned genotype.

The AoGV-E was fed to 200 neonates at one OB/ larva, which was expected to kill about 1% and at five OBs, which was expected to kill about 10%. Results closely matched these expectations (Table 4.7). Because the larvae did not die until the fifth instar it was possible to obtain sufficient virus from each larva to do some restriction digests and further infections. The larvae were harvested before they discharged virus, to obtain the maximum yield of OBs.

Table 4.7 Mortality of larvae that died infected with one or five AoGV-E OBs, in each round of *in vivo* cloning (200 larvae infected in each round).

	Infected with one OB		Infected with five OBs	
Round of cloning	No. larvae infected	% larvae infected	No. larvae infected	% larvae infected
1	1	0.5	17	8.5
2	3	1.5	24	12
3	2	1	18	9

OBs were purified individually from infected larvae and the DNA was extracted. The DNA was then digested with one or more enzymes depending on the quantity of DNA obtained. All larvae gave the same viral DNA profile as AoGV-E. In all rounds of cloning, the virus from a larva infected with one OB was used in the next round of cloning, as this was considered more homogeneous than the virus from larvae infected with five OBs. The concentration of OBs was estimated using a spectrophotometer (section 2.7.2). The virus was then diluted to the correct concentration and fed to the next round of neonates. Throughout the cloning no other genotypes were detected.

The virus from the final round of cloning was fed at an LD_{95} dose to fifty, fourth instar larvae to amplify the cloned virus, which was named AoGV-E1.

4.2.9 Comparisons of AoGV-E with other AoGV isolates

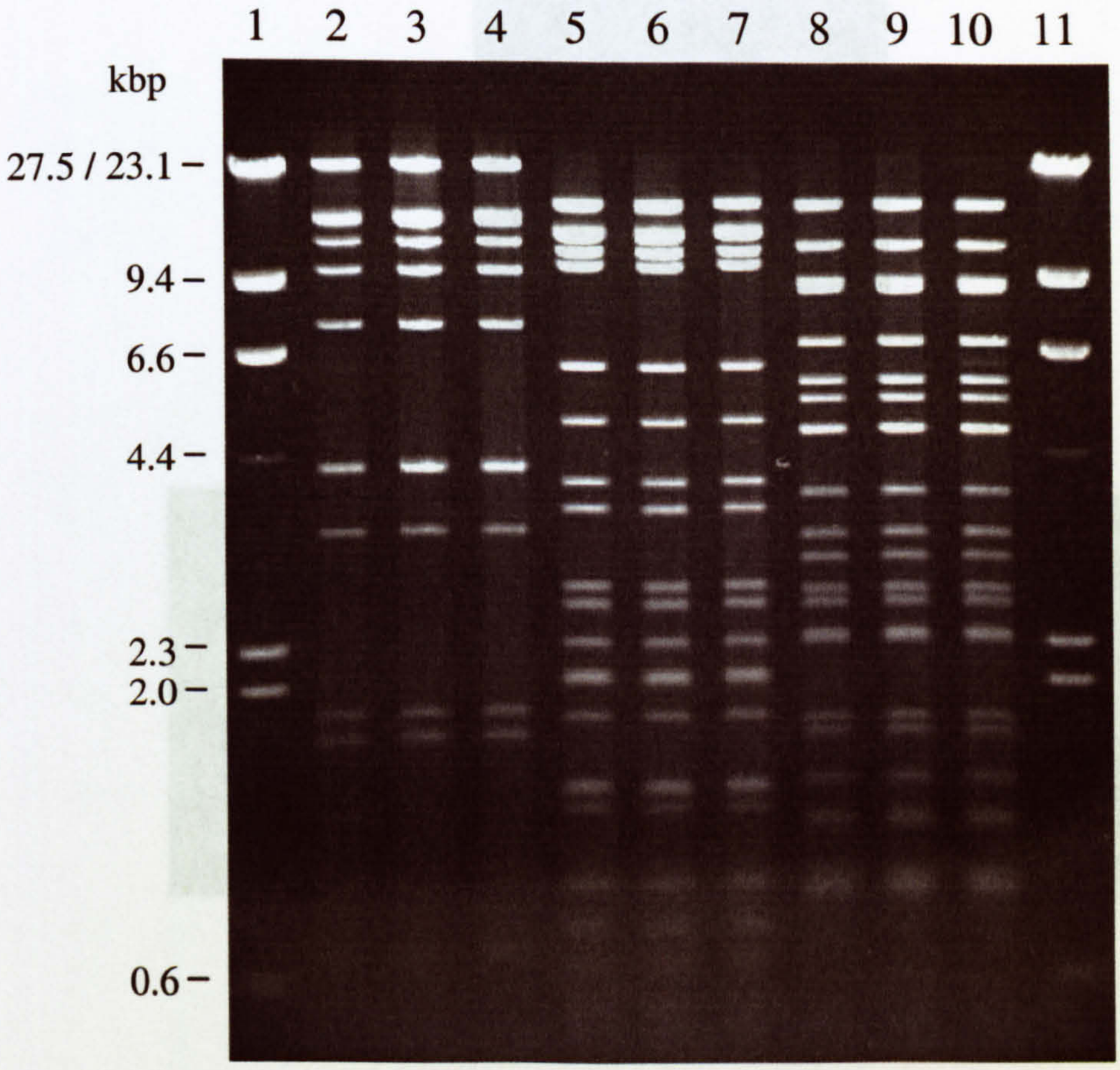
AoGV-E has the same restriction endonuclease fragment profiles (using enzymes *Bam*HI, *Eco*RI, *Hind*III and *Sal*I) as the Swiss isolate of AoGV that is used by Andermatt Biocontrol (Drolet, 1989). Virus-dead *A. orana* larvae that were collected from Italy, were provided by Dr. M. Andermatt. The larvae were all larvae/pupae intermediates similar to larvae that have died from AoGV-E. The restriction profiles of this virus were identical to AoGV-E for the six enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Pst*I, *Sac*I and *Sal*I, (Figure 4.10 and data not shown). Purified GV capsules, collected from some smaller tea tortrix larvae from Japan, were provided by Dr. Y. Kunimi (AoGV-J). This virus also had the same restriction profiles as AoGV-E for the six enzymes named above, (Figure 4.10 and data not shown). A *Sal*I restriction profile of an AoGV isolate from Japan supplied by Prof. H. Yamada to Dr. J. Drolet showed a difference in one band as compared with AoGV-S (Drolet, 1989). The fragment *Sal*I-B, which is approximately 10,932 bp in all other isolates, was absent and an extra fragment of approximately 7660 bp was present. Restriction profiles for *Bam*HI, *Hind*III and *Eco*RI were identical. Therefore, it is likely that there was at least one extra *Sal*I site in *Sal*I-B, which digested the fragment into one fragment of approximately 7660 bp and other smaller fragment(s) to the sum of 3272 bp. This is the only difference found in any of the AoGV isolates studied to date. It is interesting to note that smaller tea tortrix larvae infected with AoGV very rarely undergo incomplete pupation at the end of infection (Y. Kunimi, personal communication). However, they still do not die until final instar, regardless of when they were infected and they have similar median lethal doses (Y. Kunimi, personal communication).

4.2.10 AoNPV

The NPV that was isolated from the original infected larvae in Kent is currently being separated from the GV. This has been quite difficult due possibly to large abnormal GV OBs and aggregates of GVs that co-migrate with NPV OBs on gradients. During GV purification on glycerol gradients many OBs pelleted at the bottom of the tubes. When these were purified further and DNA extracted they were found to have the profiles of AoGV. Many abnormally large OBs were also observed during EM

Figure 4.10

Restriction endonuclease profiles of AoGV isolates from England (AoGV-E), Japan (AoGV-J) and Italy (AoGV-I). The English and Italian isolates were extracted from summer fruit tortrix larvae. The Japanese isolate was extracted from smaller tea tortrix larvae.



1+ 11 = λ *Hin*dIII

2 = AoGV-E digested with *Bgl*II

3 = AoGV-J digested with *Bgl*II

4 = AoGV-I digested with *Bgl*II

5 = AoGV-E digested with *Eco*RI

6 = AoGV-J digested with *Eco*RI

7 = AoGV-I digested with *Eco*RI

8 = AoGV-E digested with *Sal*I

9 = AoGV-J digested with *Sal*I

10 = AoGV-I digested with *Sal*I

Figure 4.11

Adoxophyes orana larvae 8 d p.i. Larvae were infected within 24 hours of reaching fourth instar with an LD_{95} dose of AoGV-E and an LD_{100} dose of AoNPV-E.

1

2

3

1 = Control larvae

2 = AoGV-E infected larvae

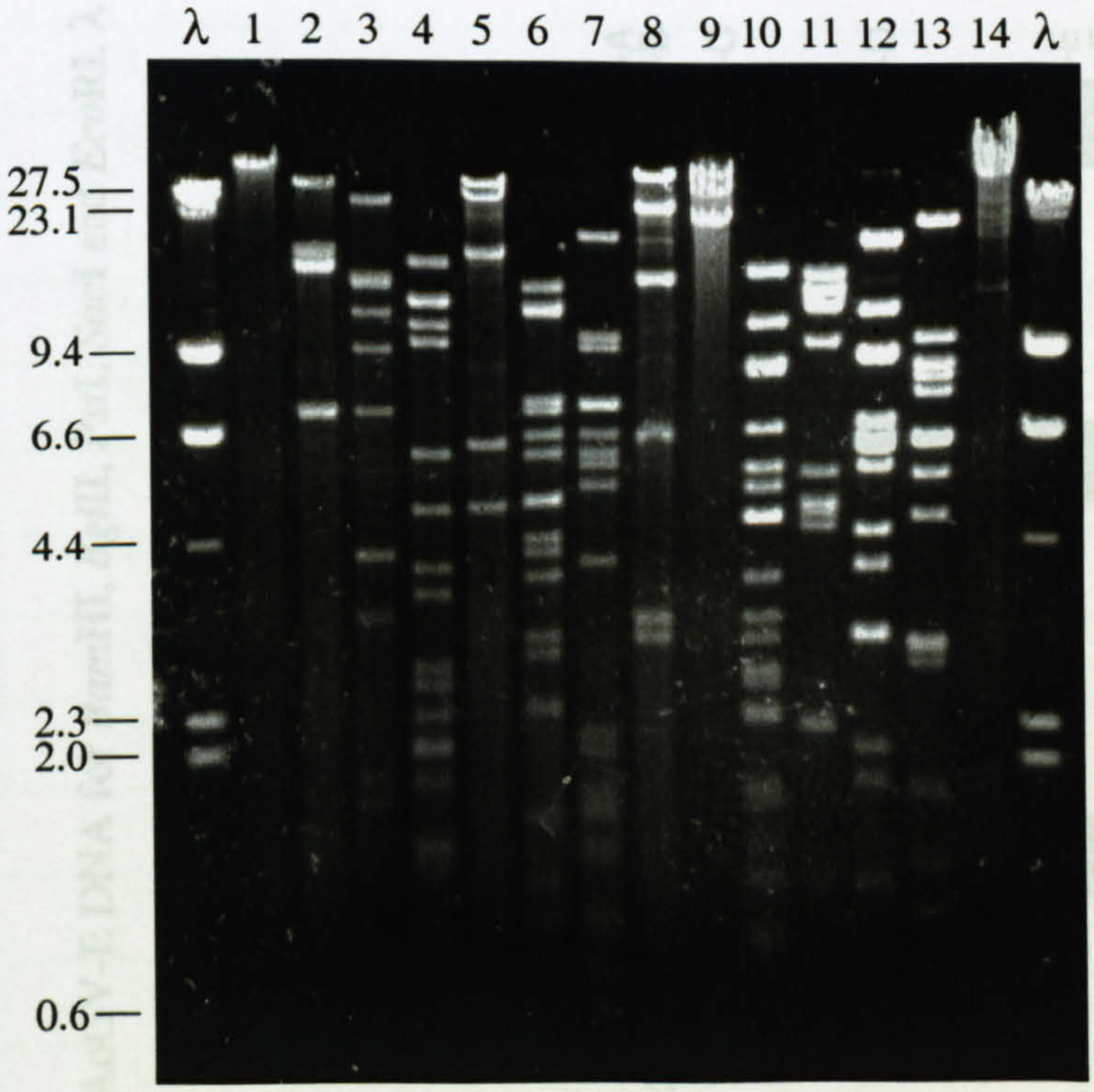
3 = AoNPV-E infected larvae

studies. OBs also appeared to be encapsulated in a matrix within the cell forming aggregates of more than 2 μm, Figure 4.9. The most pure NPV preparation that was obtained by gradient fractionation was fed to fourth instar *A. orana* larvae at doses of 10^4 - 10^6 OBs each. These larvae died typically 7-8 d p.i. The larvae died at a very small size and the virus yield was low, Figure 4.11. High doses of virus were required to establish infection. Fifth instar larvae appeared almost resistant to NPV infection. Attempts to clone the NPV by limiting dilution always resulted in predominantly GV-infected larvae.

4.2.11 Construction of a physical map of the AoGV-E genome

The AoGV-E genome was digested with 14 restriction enzymes, Figure 4.12. Digests with *Bam*HI, *Bgl*II, *Pst*I and *Sac*I were chosen for analysis, which gave a range in the number of fragments from 3 to 12. This would make the initial map less complicated to construct and the map could then be added to later choosing enzymes that produced a larger number of fragments.

Fig. 4.12 Restriction endonuclease fragment profiles of AoGV-E run on a 0.7% agarose gel. λ *Hin*dIII (λ) standards were used. Restriction digests of *Kpn*I, *Pst*I and *Xho*I have submolar bands as a result of the DNA being partially digested or over digested.



Restriction endonuclease profiles of singly digested AoGV-E with either *Bam*HI, *Bgl*II, *Pst*I, *Sac*I and *Eco*RI are shown in Figure 4.13. Double digests were performed using each pair of restriction enzymes used for the initial mapping e.g. *Bam*HI and *Bgl*II, Figure 4.14.

Figure 4.13
Restriction enzyme profiles of AoGV-E DNA for *Bam*HI, *Bgl*II, *Pst*I, *Sac*I and *Eco*RI. λ DNA digested with *Hin*dIII is included for molecular size standards

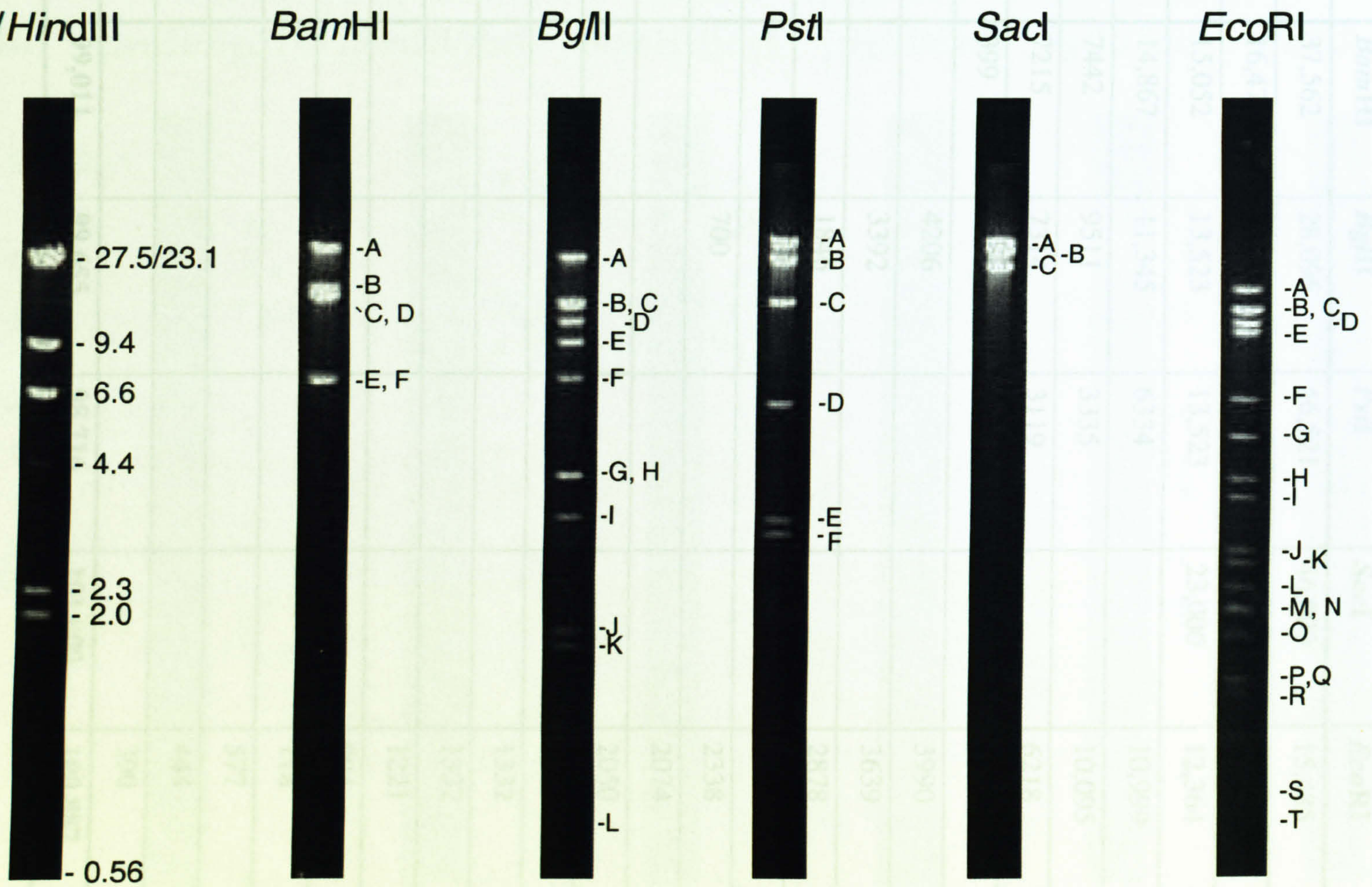


Table 4.8 Estimated sizes in bp of restriction fragments of singly digested AoGV-E DNA. Fragments are labelled in order of decreasing size, with **A** for the largest fragment.

Fragment	*Bam*HI	*Bgl*II	*Pst*I	*Sac*I	*Eco*RI
A	37,562	28,096	46,521	46,000	15,485
B	16,474	13,838	25,884	32,000	12,364
C	15,052	13,523	13,523	23,000	12,364
D	14,867	11,345	6334		10,999
E	7442	9511	3335		10,095
F	7215	7314	3119		6218
G	399	4206			5013
H		4206			3990
I		3392			3639
J		1846			2878
K		1678			2691
L		700			2338
M					2074
N					2050
O					1771
P					1332
Q					1332
R					1221
S					904
T					718
U					577
V					444
W					390
TOTAL	**99,011**	**99,655**	**98,716**	**101,000**	**100,887**

Figure 4.14
Restriction enzyme profiles of AoGV-E DNA for *Bam*HI, *Bgl*II, *Pst*I and *Sac*I pairwise double digests. λ DNA digested with *Hin*dIII is included for molecular size standards

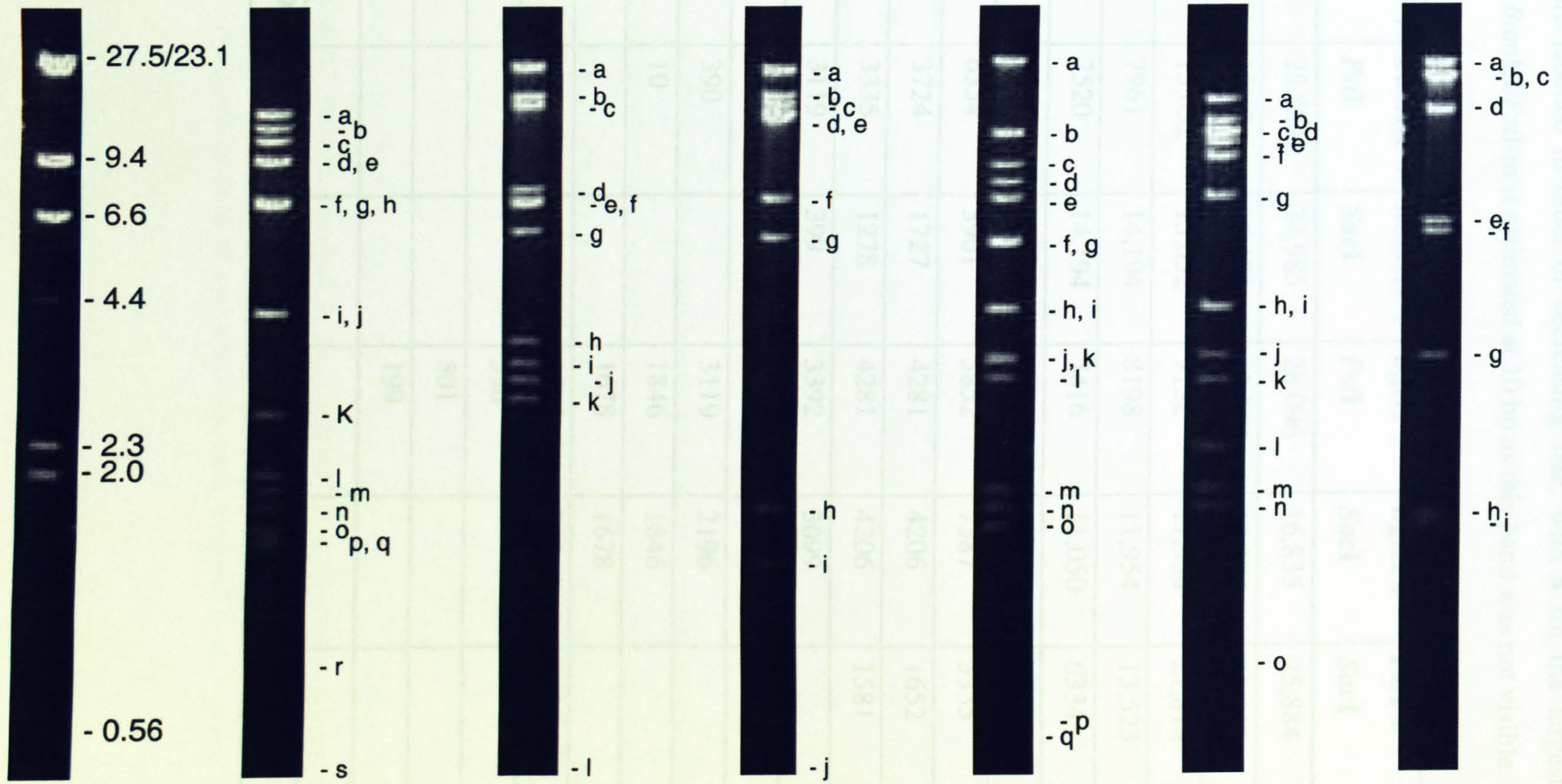


Table 4.9 Estimated sizes in bp of restriction fragments of doubly digested AoGV-E DNA. Fragments are labelled in order of decreasing size, with **a** for the largest fragment. Fragment *Bam*HI/*Pst*I-m is estimated at 10 bp as this band was not visible.

Fragment	*Bam*HI + *Bgl*II	*Bam*HI + *Pst*I	*Bam*HI + *Sac*I	*Bgl*II + *Pst*I	*Bgl*II + *Sac*I	*Pst*I + *Sac*I
a	13,523	25,884	24,985	28,096	16,835	25,884
b	12,021	16,474	16,474	11,563	13,275	21,818
c	11,135	15,052	15,052	9132	11,954	21,818
d	9511	7961	14,194	8198	11,954	13,523
e	9511	7520	14,194	7416	11,050	6334
f	7417	7314	7387	5852	9719	5901
g	7317	6334	5901	5852	7387	3335
h	7294	3724	1727	4281	4206	1652
i	4243	3335	1278	4281	4206	1581
j	4243	3119	399	3392	3099	
k	2664	2870		3335	2908	
l	1981	390		3119	2196	
m	1846	10		1846	1846	
n	1678			1678	1678	
o	1600			1522	700	
p	1504			596		
q	1502			501		
r	700			199		
s	399					
TOTAL	**100,089**	**99,987**	**101,591**	**100,859**	**103,013**	**101,846**

The size of each fragment from the single and double digestions were estimated using size standards λ *Hin*dIII, 1 kb ladder and High Molecular Weight DNA Markers. (section 2.2.4). These sizes are shown in Tables 4.8 and 4.9.

When interpreting the double digestions, two assumptions could be made:

1) Bands from a single digestion which were still present after a double digestion did not contain the restriction site of the secondary enzyme.

2) Given the DNA was circular, the number of fragments produced from two single digestions had to equal the number of fragments from the equivalent double digestion.

Using this information, certain deductions could be made, such as *Bam*HI-B did not have a *Sac*I site. Also, it is likely that *PstI*/*Sac*I -a (25,884 kbp) and –f (5901 kbp) form *Sac*I –B (32,000 kbp). The double digest fragments produced from each restriction fragment are tabulated in Appendix 5.

To gain more information about the position of fragments relative to each other within the map, specific fragments were used as probes in Southern blot analyses of singly and doubly digested AoGV-E. Figure 4.15 shows an example of this analysis. The restriction endonuclease fragments of AoGV-E used as probes are shown in Table 4.10.

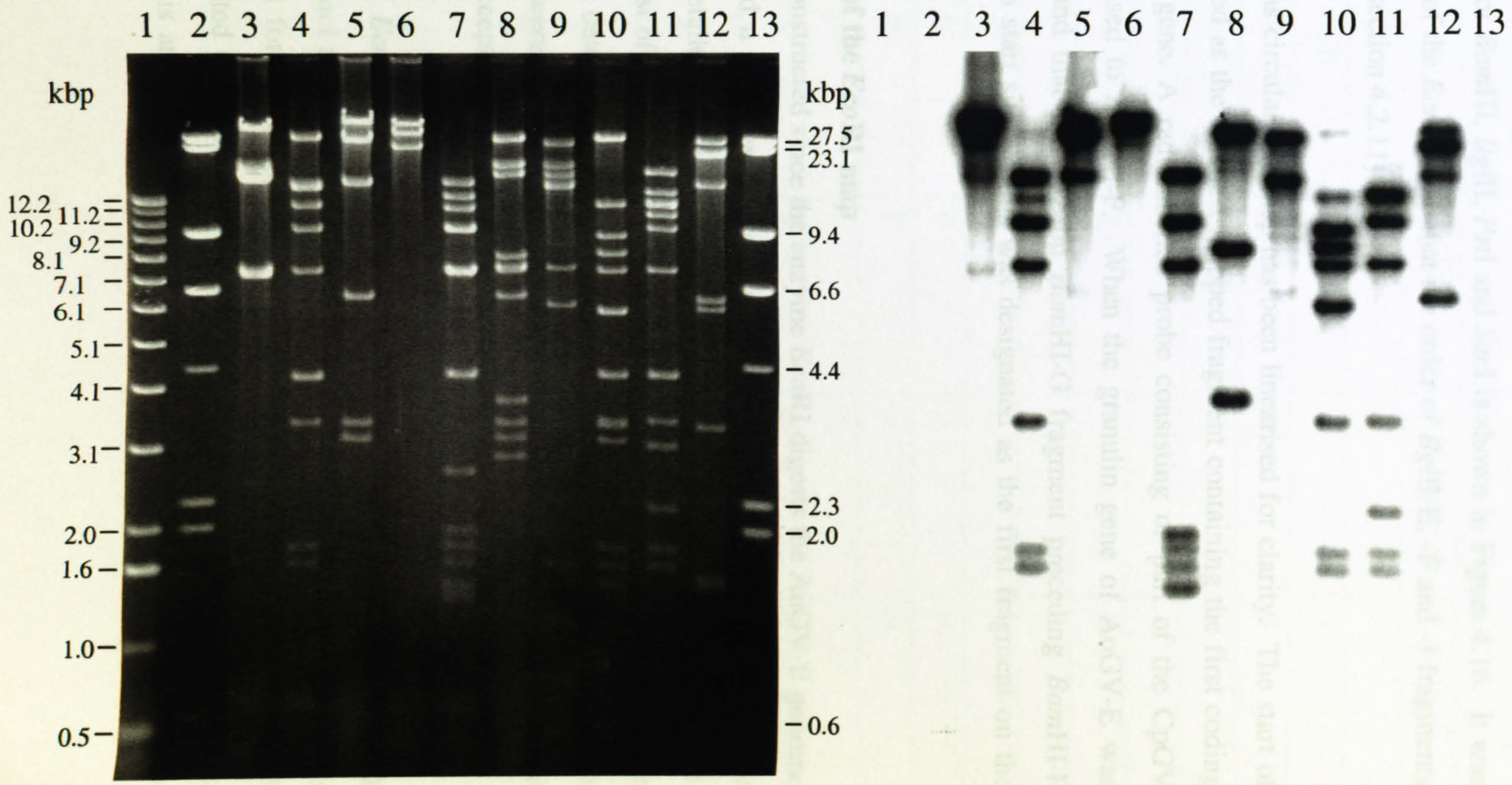
The hybridisation results and double digest information was used to calculate the positions of the fragments. All the data had to be incorporated to construct a map of overlapping fragments which agreed with every hybridisation result and every double digest fragment size. All combinations of two enzymes were mapped first, Appendix 6.

Table 4.9
The restriction fragments of AoGV-E that hybridise to AoGV-E radiolabelled probes at high stringency (65°C). Letters in parenthesis indicate low hybridisation. Letters which have no spaces between them indicate comigrating fragments of which either/any could be the hybridising fragment. Lines within boxes indicate that fragments did hybridise but were not required for the mapping of the genome.

Probes	**Restriction fragments that hybridised**									
	***Bam*HI**	***Bgl*II**	***Pst*I**	***Sac*I**	***Bam*HI+ *Bgl*II**	***Bam*HI + *Pst*I**	***Bam*HI + *Sac*I**	***Bgl*II + *Pst*I**	***Bgl*II + *Sac*I**	***Pst*I + *Sac*I**
***Bam*HI-A**	A	BC, (D), E, F, I, J, K	A, B, C	A, B	a, de, fgh, l, m, n, o	a, d, h	a, de	_____	_____	_____
***Bgl*II-A**	BD, EF, G	A	A	B, C	c, de, h, s	_____	_____	a	a, b	_____
***Pst*I-A**	(A), B, CD, EF, G	A, BC, GH, I	A	B, C	_____	b, c, d, ef, l	_____	a, fg, hi, jk, o	_____	a, bc
***Sac*I-A**	A, C, EF	BC, D, E, F, J, K	B, C, D, E, (F)	A	_____	_____	a, de, f	_____	cd, e, f, g, l, m, n, o	bc, d, e, g, h
***Bam*HI-EF**	EF	A, D	A, C	A, (B), C	fgh	ef	f, g, i	_____	_____	
***Bgl*II-D**	A, CD, EF	D	C	A	fgh, k, o	_____	_____	b	cd	_____
***Pst*I-B**	A	BC, E, F, J	B	A, B	_____	a	_____	c, d, e, m	_____	bc, f

Figure 4.15
Left panel: 0.7 % agarose gel of various restriction digests of AoGV-E.
Right panel: Autoradiograph of the gel probed with AoGV-E fragment *Bam*HI-A at high stringency (65°C).

1= kb ladder
2 + 13 = λ *Hin*dIII
3 = *Bam*HI
4 = *Bgl*II
5 = *Pst*I
6 = *Sac*I
7 = *Bam*HI + *Bgl*II
8 = *Bam*HI + *Pst*I
9 = *Bam*HI + *Sac*I
10 = *Bgl*II + *Pst*I
11 = *Bgl*II + *Sac*I
12 = *Pst*I + *Sac*I



There were still some areas of ambiguity. *Bgl*II-L had not been placed, *Pst*I-D and -E could be either way round and *Bgl*II-E, -F and -J could be in any order. Fragment *Bgl*II-L was cloned into the *Bam*HI site of pBSK+ to create an additional probe which hybridised to *Bam*HI-A, *Pst*I-D and –C and *Sac*I-A. This placed *Bgl*II-L between *Bgl*II-C and –D. It also determined the order of *Pst*I-D and -E as it hybridised to *Pst*I-D and –C. The map for *Bam*HI, *Bgl*II, *Pst*I and *Sac*I is shown in Figure 4.16. It was not until construction of the *Eco*RI map that the order of *Bgl*II-E, -F and -J fragments could be determined (section 4.2.11).

Although the DNA was circular, the map has been linearised for clarity. The start of the map was designated as the smallest mapped fragment containing the first coding base of the granulin gene. A radiolabelled probe consisting of part of the CpGV granulin gene hybridised to *Bam*HI-F. When the granulin gene of AoGV-E was sequenced it was found that the 362 bp *Bam*HI-G fragment preceding *Bam*HI-F contained the granulin start site, so this was designated as the first fragment on the map.

4.2.12 Construction of the *Eco*RI map

An *Eco*RI map was constructed since the enzyme *Eco*RI digests the AoGV-E genome into 23 fragments and a more extensive map would aid in the sequencing of the genome and a more detailed comparative analysis of AoGV-E to other baculoviruses. Firstly, an *Eco*RI digest of AoGV-E DNA was analysed by gel electrophoresis and the fragment sizes were determined (section 2.2.4), Table 4.11. AoGV fragments digested with *Eco*RI were then shotgun cloned into *Eco*RI digested pBSK+ (section 2.3). All fragments except A, C and O were successfully cloned.

The cloned AoGV-E *Eco*RI fragments were then used to probe blots containing *Bam*HI, *Bgl*II, *Pst*I, *Sac*I and *Eco*RI digests of AoGV-E. Fragments not obtained as clones were obtained for use as probes directly from *Eco*RI digests of AoGV-E genomic DNA separated on agarose gels. The bands of DNA that hybridised were recorded and from this an estimate of the position of the fragment could be made, Table 4.11.

Table 4.11 Estimated sizes and Southern blot hybridisation data summary for AoGV-E *Eco*RI fragments. Letters in parentheses indicate low hybridisation.

		Hybridising fragments from digests			
AoGV-E *Eco*RI fragment	Size (bp)	*Bam*HI fragments	*Bgl*II fragments	*Pst*I fragments	*Sac*I fragments
A	15,485	C, D	C, (D)	A, C, D, E, F	A, C
B	12,364	D	A, G	A	C
C	12,364	A, B	A, B, H, I	A	B
D	10,999	B	A	A	B
E	10,095	A, C, E	D	C	A
F	6218	A	B, F, J	B	A
G	5013	A	E	B	A
H	3990	F	A	A	C
I	3639	A	E, K	B, C	A
J	2878	A	B	B	B
K	2691	A	F	B	A
L	2338	A	B	B	A, B
M	2074	A	E	B	A
N	2050	D, F	A	A	C
O	1771	A	F	B	A
P	1322	A	B	A	B
Q	1322	A	B	A	B
R	1221	D	C, G	A	C
S	904	A	B	A, B	B
T	718	F	A	A	B
U	577	A	B	A	B
V	444	A	E	B	A
W	390	F	A	A	C

Most fragments could be satisfactorily placed on the physical map using this method but certain areas needed more investigation. These included areas in which multiple fragments hybridised to the same areas on the initial map, thus making it difficult to order these fragments. For example, *Eco*RI- M, -V and -G all hybridised to *Bam*HI-A, *Bgl*II-E, *Pst*I-B and *Sac*I-A. Also, *Eco*RI -P, -Q and -U all hybridised to *Bam*HI-A, *Bgl*II-B ,*Pst*I-A and *Sac*I-B. To order these fragments, partial digests of an excised fragment containing all of the grouped fragments needed to be performed. Because of the difficulty in recovering enough DNA to perform partial digests from gels of digests of the AoGV-E genomic DNA, it was decided to try and clone the smallest fragment of the genome that contained these *Eco*RI fragments.

4.2.12.1 Ordering of *Eco*RI-M, -V and -G

The smallest fragment containing *Eco*RI-M, -V and -G was *Bgl*II-E, which was 9511 bp. This fragment was cloned into the *Bam*HI site of pBSK+. From the hybridisations described above, it was known that *Eco*RI–I hybridised to *Bgl*II-K and –E. This meant that *Bgl*II–E contained part of *Eco*RI-I on its left hand side. This allowed the orientation of the clone to be determined. The clone was digested with *Pst*I to release the *Pst*I/*Bgl*II-c fragment of 9132 bp which contained part of *Eco*RI-I and all of *Eco*RI-M, -V and -G. This was then purified from a gel. A Southern blot of AoGV digests was probed with *Bgl*II-E and confirmed that it hybridised to the correct fragments, *Eco*RI–I, -G, -M and V. The DNA was then digested with *Eco*RI and the fragments of 5.0, 2.1, 1.6, and 0.45 kbp were obtained.

From the hybridisations it had been established that *Bgl*II-E hybridised to part of *Eco*RI-I on the left hand side. This portion of *Eco*RI-I was assumed to be the 1.6 kbp fragment as the 5.0, 2.1 and 0.45 kbp fragments corresponded in size to the G, M and V fragments respectively. In a partial digest of *Bgl*II-E with *Eco*RI, one of six outcomes was to be predicted. The fragments in all outcomes would include each of the complete digest fragments (5.0 kbp, 2.1 kbp, 1.6 kbp and 0.45 kbp) and completely undigested DNA of 9.1 kbp. The other possible partial digests are shown in Table 4.12 .

Table 4.12 Partial digest fragment sizes predicted for various combinations of *Eco*RI fragments within *PstI/Bgl*II-c.

	I+M 3.6 kbp	I+V 2.0 kbp	I+G 6.6 kbp	M+V 2.4 kbp	M+G 7.0 kbp	G+V 5.4 kbp	M+V+G 7.4 kbp	I+M+V 4.0 kbp	I+M+G 8.6 kbp	I+G+V 7.0 kbp
I-M-V-G	✓			✓		✓	✓	✓		
I-V-M-G		✓		✓	✓		✓	✓		
I-M-G-V	✓				✓	✓	✓		✓	
I-V-G-M		✓			✓	✓	✓			✓
I-G-M-V			✓	✓	✓		✓		✓	
I-G-V-M			✓	✓		✓	✓			✓

When a partial *Eco*RI digest was performed on *PstI/Bgl*II-c along with the expected fragment sizes of 9.1, 5.0, 2.1, 1.6 and 0.45 kbp, the bands in Table 4.13 were produced.

Table 4.13 Partial digest fragment sizes obtained for *PstI/Bgl*II-c digested with *Eco*RI.

	I+M 3.6 kbp	I+V 2.0 kbp	I+G 6.6 kbp	M+V 2.4 kbp	M+G 7.0 kbp	G+V 5.4 kbp	M+V+G 7.4 kbp	I+M+V 4.0 kbp	I+M+G 8.6 kbp	I+G+V 7.0 kbp
Partial digest		✓			✓	✓	✓			✓

This result confirmed the *Eco*RI fragment order was **I-V-G-M.**

4.2.12.2 Ordering of *Eco*RI -P, -Q and -U

The smallest fragment containing *Eco*RI -P, -Q and -U was *Bgl*II/*Pst*I –g, which is 5852 bp. This fragment has not yet been cloned. However, from other evidence, some information about the order could be obtained.

From the hybridisation data, it was known that *Bgl*II/*Pst*I-g comprised parts of *Eco*RI-S and *Eco*RI-C on its left and right end respectively, which flanked the fragments *Eco*RI-P, Q and U in an order to be determined. From a double digestion of AoGV with *Pst*I and *Eco*RI, *Eco*RI-S was digested into two fragments -χ (321 bp) and -y (587 bp), Appendix Table 7.1. As *Eco*RI-S gave a stronger hybridisation signal to *Pst*I – A than *Pst*I – B, it was predicted that the larger fragment -y was within *Pst*I – A. This will have to be checked by digesting *Bgl*II/*Pst*I –g once it is cloned. Therefore, the predicted size of the portion of the *Eco*RI-C fragment lying within *Bgl*II/*Pst*I –g is;

5852 bp (*Bgl*II/*Pst*I –g) – 587 bp (Part of *Eco*RI-S) – 1332 bp *(Eco*RI-P) – 1332 bp (*Eco*RI-Q) – 577 bp (*Eco*RI-U) = **2024 bp**.

These inferences allowed prediction of the possible partial digest products of *Eco*RI on *Bgl*II/*Pst*I-g, Table 4.14. Because of similarities in size between some of the complete *Eco*RI digestion products of this fragment, multiple combinations make up the same fragment size. In all events, product sizes of undigested DNA (5.9 kbp), S-P-Q-U in any order (3.8 kbp), P-Q-U-C in any order (5.3 kbp), P-Q-U in any order (3.2 kbp) and complete digest products (2.0 kbp, 1.3 kbp and 0.6 kbp) were expected, together with some characteristic partial digest fragments.

Table 4.14 Partial digest fragment sizes predicted for various combinations of *Eco*RI fragments within *Bgl*II/*Pst*I –g.

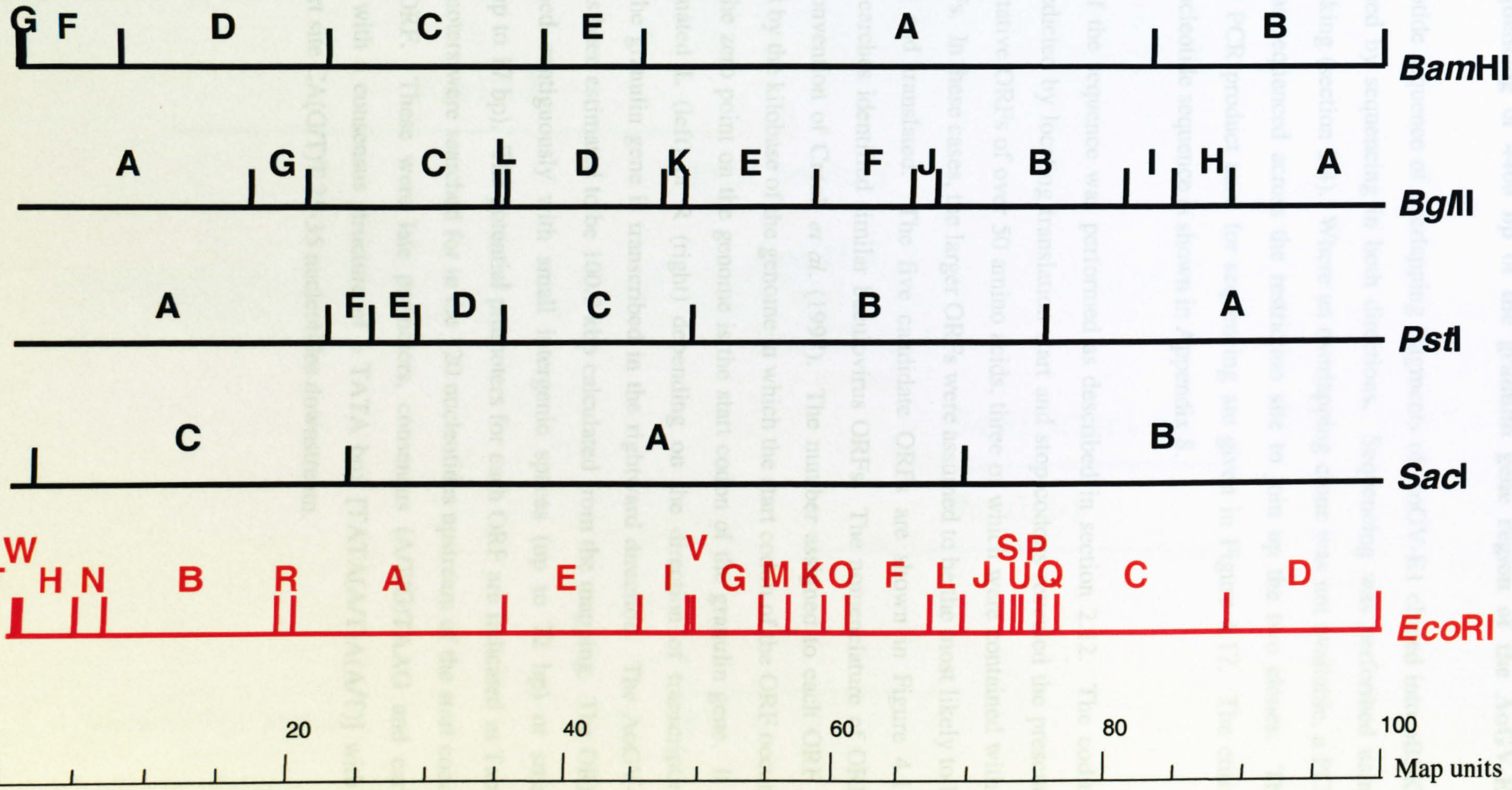
	S+P/ S+Q/ S+U/ P+U/ Q+U	P+Q	P+C/ Q+C	U+C	S+P+Q	S+Q+U/ S+P+U	Q+U+C /P+U+C	P+Q+C
	1.9 kbp	**2.7 kbp**	**3.4 kbp**	**2.6 kbp**	**3.3 kbp**	**2.5 kbp**	**3.9 kbp**	**4.7 kbp**
S-P-Q-U-C	✓	✓		✓	✓		✓	
S-P-U-Q-C	✓		✓			✓	✓	
S-Q-P-U-C	✓	✓		✓	✓		✓	
S-Q-U-P-C	✓		✓			✓	✓	
S-U-P-Q-C	✓	✓	✓			✓		✓
S-U-Q-P-C	✓	✓	✓			✓		✓

To perform this analysis, 125 µg AoGV DNA was digested with *Pst*I and *Bgl*II and fragment mixture -fg was excised and purified. It did not matter that -f was present as it was known not to contain any *Eco*RI sites. A partial digest with *Eco*RI was attempted with the small amount of DNA recovered from the excised fragment. Three bands were produced. One band was 5.9 kbp, which was *Bgl*II/*Pst*I –f and possibly undigested *Bgl*II/*Pst*I –g. A band of 4.7 kbp and a double band at 0.6 kbp were also produced. Although not all partial digest products were produced, this information did clarify the order of fragments, as the only way a 4.7 kbp fragment could be produced was if the order was S-U-Q-P-C or S-U-P-Q-C. As it was now clear that -P was adjacent to -Q and that they were the same size, their order was not important. Therefore, -P was placed to the left of –Q. Discrimination between *Eco*RI-P and Q will require characterisation of individual clones of these fragments. Therefore, the *Eco*RI order within *Bgl*II/*Pst*I –g was **S-U-P-Q-C.** This completed the ordering of the *Eco*RI map, Figure 4.16.

To further specify the positioning of the *Eco*RI fragments in relation to the fragments produced by the other mapped enzymes, double digests were performed using *Eco*RI and each of the four previously mapped enzymes (Appendix Table 7.1). The sum of

the sizes of smaller, double digest fragments were used to obtain a more accurate estimate of the sizes of the single digest fragments. These data are available in Appendix Table 7.2.

Figure 4.16
Restriction endonuclease map of AoGV-E for the enzymes *Eco*RI, *Bam*HI, *Bgl*II, *Pst*I and *Sac*I. The zero point for the map is the start of the granulin gene. The *Eco*RI map is coloured red because only the order of fragments has been established not the relationship to the other restriction sites.



4.2.13 Sequencing of 4460 bp of the granulin gene region of the AoGV-E1 genome

The nucleotide sequence of overlapping fragments of AoGV-E1 cloned into pBSK+ was obtained by sequencing in both directions. Sequencing was performed using primer walking (section 2.4). Where an overlapping clone was not available, a PCR product was sequenced across the restriction site to join up the two clones. The clones and PCR product used for sequencing are given in Figure 4.17. The entire 4460 bp nucleotide sequence is shown in Appendix 8.

Analysis of the sequence was performed as described in section 2.12. The coding regions predicted by locating translation start and stop codons revealed the presence of eight putative ORFs of over 50 amino acids, three of which were contained within larger ORFs. In these cases, the larger ORFs were assumed to be the most likely to be transcribed and translated. The five candidate ORFs are shown in Figure 4.18. Database searches identified similar baculovirus ORFs. The nomenclature of ORFs uses the convention of Crook *et al.* (1997). The number assigned to each ORF is determined by the kilobase of the genome in which the start codon of the ORF occurs; assuming the zero point on the genome is the start codon of the granulin gene. It is then designated L (left) or R (right) depending on the direction of transcription assuming the granulin gene is transcribed in the rightward direction. The AoGV-E genome has been estimated to be 100.9 kbp calculated from the mapping. The ORFs were aligned contiguously with small intergenic spaces (up to 72 bp) or small overlaps (up to 17 bp). The potential promoters for each ORF are indicated in Table 4.15. Promoters were searched for in the 120 nucleotides upstream of the start codon for each ORF. These were late promoters, consensus (A/T/G)TAAG and early promoters with a consensus structure of a TATA box [TATA(A/T)A(A/T)] with a mRNA start site CA(G/T)T 25-35 nucleotides downstream.

Figure 4.17

AoGV-E1-containing plasmids and PCR product used to sequence the granulin-containing region of AoGV-E1. Red blocks represent regions of the plasmid or PCR product that were sequenced in both directions.

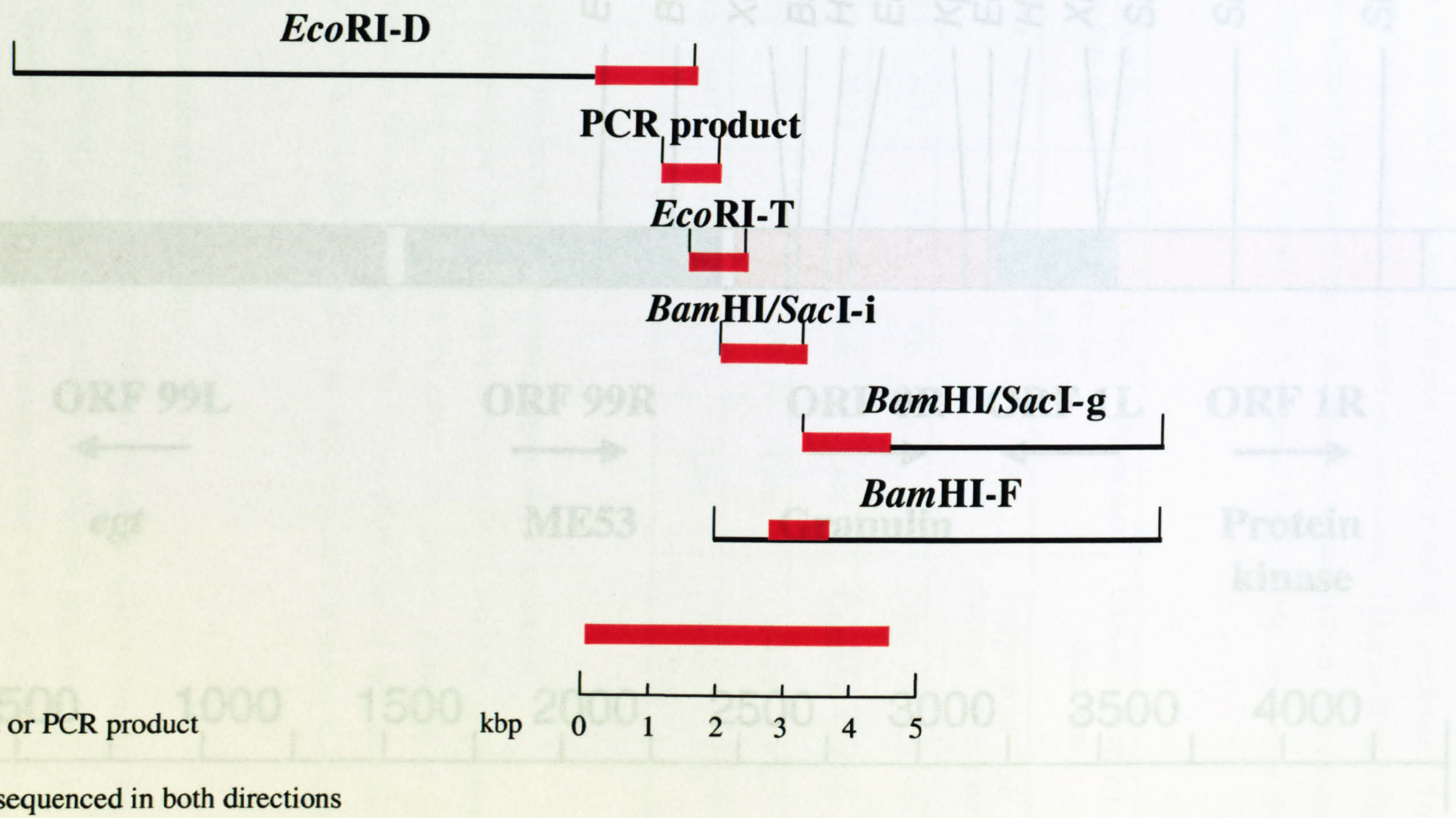


Figure 4.18. Restriction map and ORFs identified in the 4460 bp sequence of the AoGV-E1 granulin-containing region. Arrows indicate the direction of transcription.

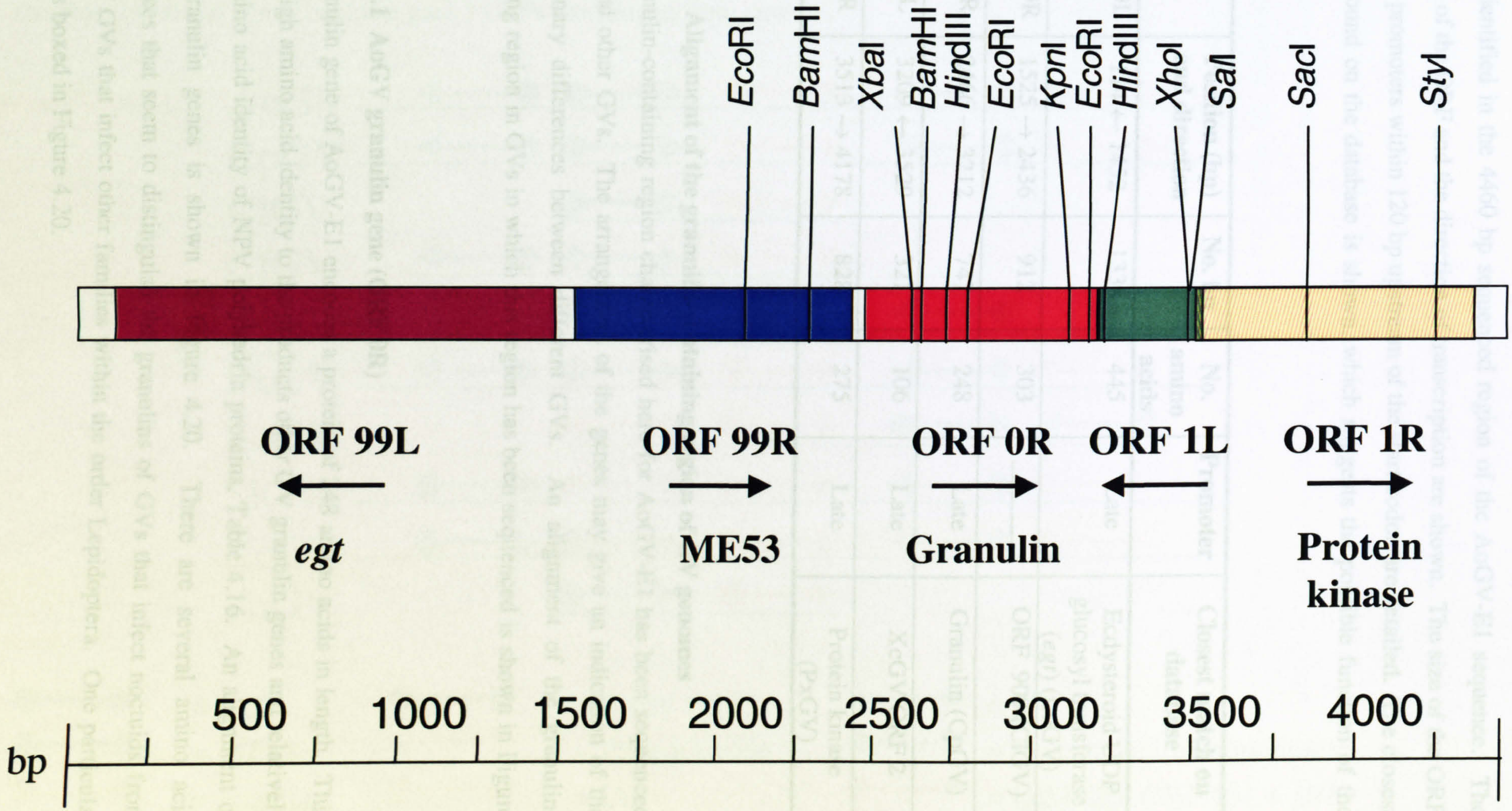


Table 4.15

ORFs identified in the 4460 bp sequenced region of the AoGV-E1 sequence. The position of the ORF and the direction of transcription are shown. The size of the ORF and any promoters within 120 bp upstream of the start codon are detailed. The closest match found on the database is shown, which suggests the possible function of the ORF.

ORF	Position (bp) and direction	No. bp	No. amino acids	Promoter	Closest match on database
ORF 99L	114 ← 1452	1338	445	Late	Ecdysteroid UDP glucosyl transferase (*egt*) (LoGV)
ORF 99R	1525 → 2436	912	303	_____	ORF 909 (ClGV)
ORF 0R	2466 → 3212	747	248	Late	Granulin (CpGV)
ORF 1L	3209 ← 3529	321	106	Late	XcGV ORF 2
ORF 1R	3513 → 4178	828	275	Late	Protein kinase (PxGV)

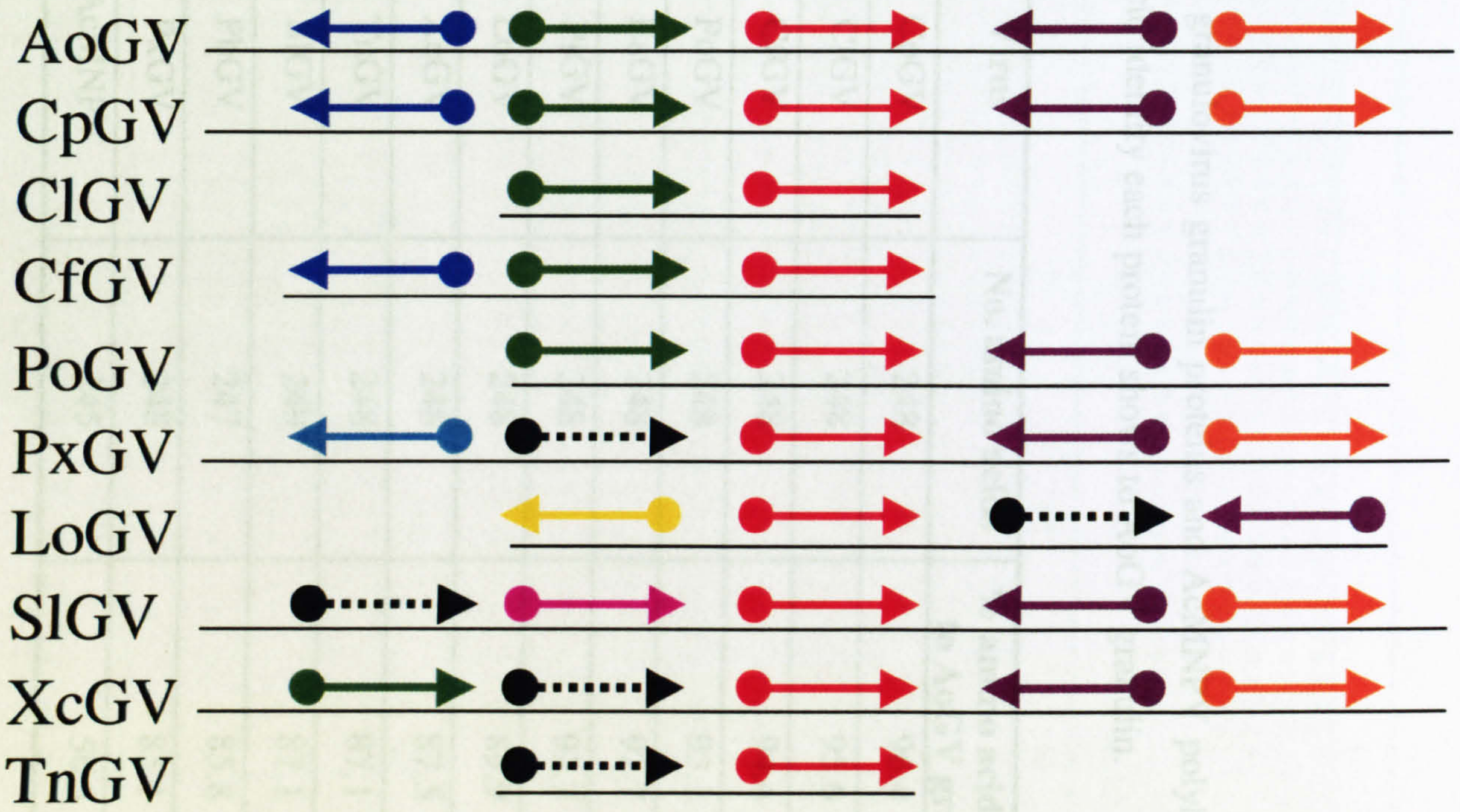
4.2.13.1 Alignment of the granulin-containing region of GV genomes

The granulin-containing region characterised here for AoGV-E1 has been sequenced in several other GVs. The arrangement of the genes may give an indication of the evolutionary differences between different GVs. An alignment of the granulin-containing region in GVs in which this region has been sequenced is shown in Figure 4.19.

4.2.13.2.1 AoGV granulin gene (ORF 0R)

The granulin gene of AoGV-E1 encodes a protein of 248 amino acids in length. This shows high amino acid identity to the products other GV granulin genes and relatively high amino acid identity of NPV polyhedrin proteins, Table 4.16. An alignment of some granulin genes is shown in Figure 4.20. There are several amino acid differences that seem to distinguish the granulins of GVs that infect noctuids from those of GVs that infect other families within the order Lepidoptera. One particular region is boxed in Figure 4.20.

Figure 4.19 Comparison of the granulin containing regions of various GVs. The ORFs are all shown as the same size and evenly spaced for clarity. Spaces indicate unsequenced DNA. PxGV contains a ME53 homologue 4 ORFs upstream of granulin and an *egt* gene 6 ORFs upstream of granulin (Hashimoto, 1999). LoGV contains an *egt* gene 8 kbp upstream from granulin (Smith and Goodale, 1998) and a protein kinase gene 3 ORFs downstream from granulin.



= EGT
= ME53
= granulin
= XcGV ORF 2
= Protein kinase
= IAP
= p10
= No baculovirus homologue
= LdMNPV ORF 151 + 162

Phylogenetic analysis of granulin proteins is shown in Figure 4.21. These analyses distinguished two basic subgroups. One subgroup comprised of granulins from slow noctuid-infecting GVs and the other from fast GVs that infect other families. LoGV and PxGV did not group strongly to either group. AoGV-E1 groups with other tortricid infecting GVs. The GVs appear to group depending on the family of insect they infect rather than the tissue tropism of the infection or speed of kill. A better interpretation of this observation will be possible when more GV granulin sequences are available.

Table 4.16

The lengths of granulovirus granulin proteins and AcMNPV polyhedrin protein and the % amino acid identity each protein shows to AoGV granulin.

Virus	No. amino acids	% amino acid identity to AoGV granulin
CpGV	248	96.4
ClGV	248	95.6
CfGV	248	94.4
PoGV	248	93.1
EaGV	248	92.7
PiGV	248	92.7
LoGV	248	89.9
XcGV	248	87.5
TnGV	248	87.1
SlGV	249	87.1
PbGV	247	85.8
PxGV	248	85.1
AcMNPV	245	56.3

Figure 4.20

Multiple alignment of the amino acid sequences of the granulin proteins of GVs. Common regions are shaded pink. A region which differs in noctuid-infecting GVs as compared to GVs which infect other families of Lepidoptera is boxed.

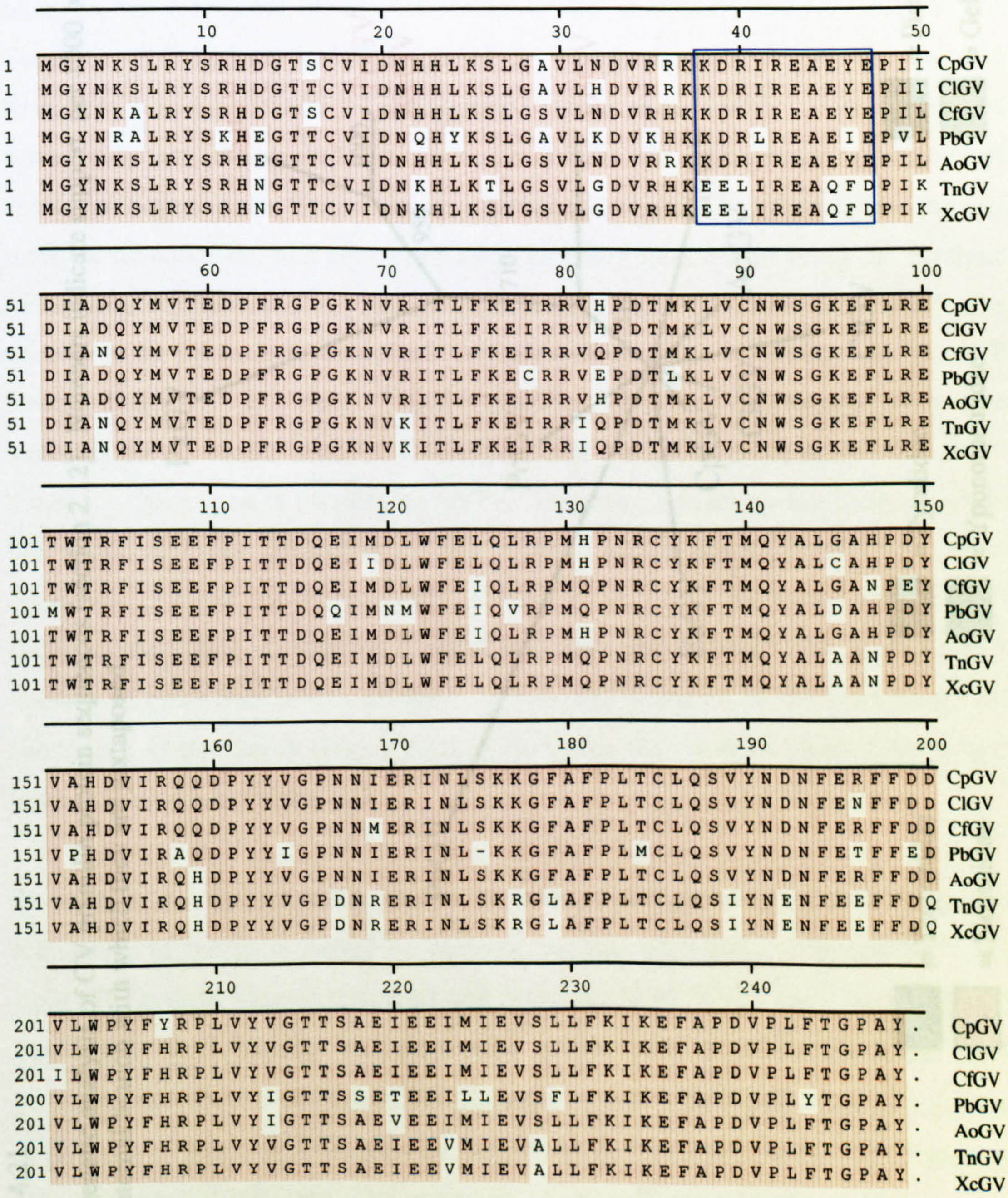
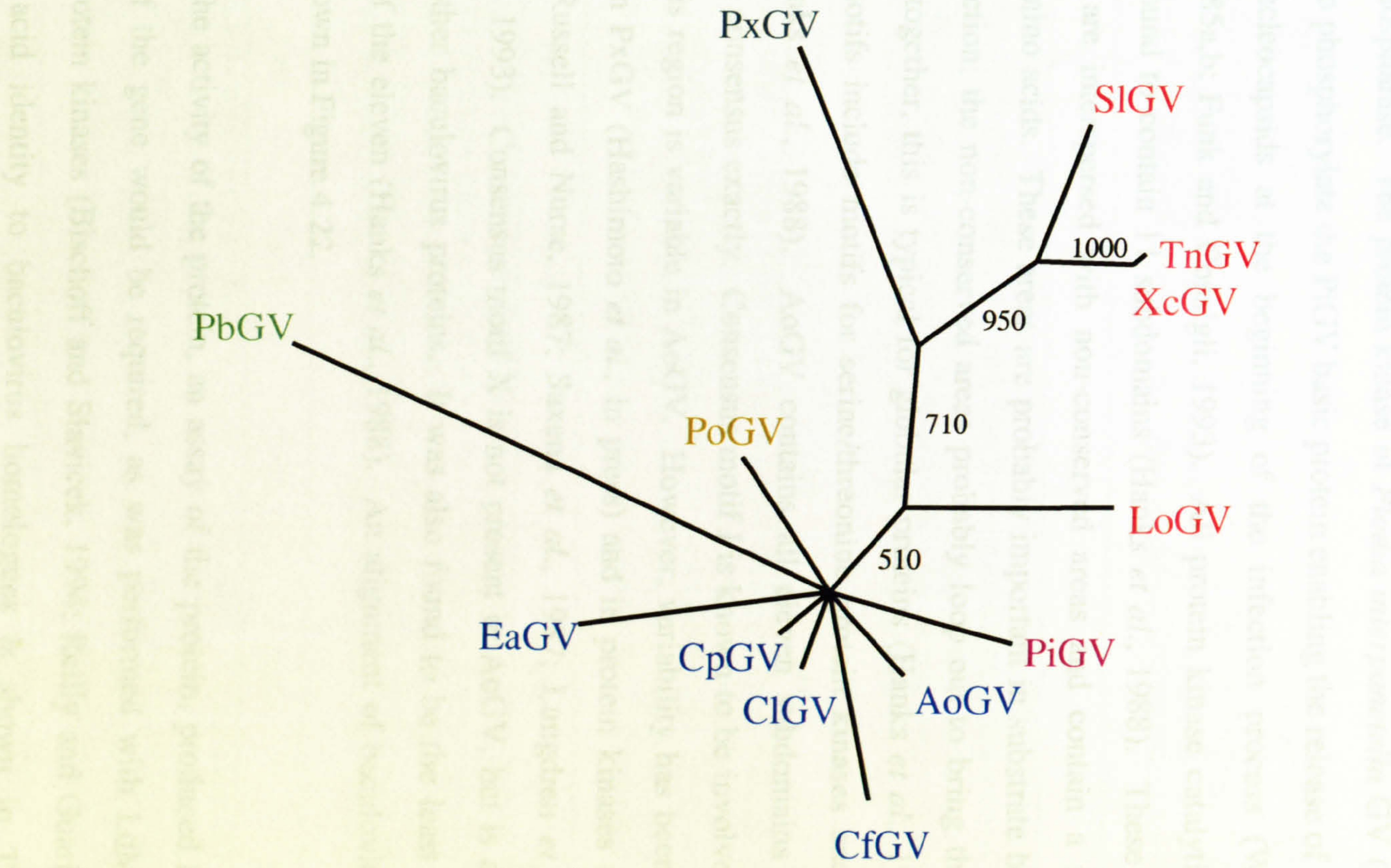


Figure 4.21

Phylogenetic analysis of GV granulin protein sequences (section 2.12). Numbers indicate support, per 1000 bootstrap replications, for each internal branch with which they are juxtaposed.



4.2.13.3 Protein kinase (ORF 1R)

Another gene found in the sequenced region of AoGV-E and other baculoviruses encodes a putative protein kinase. Protein kinases are enzymes that specifically add phosphate groups to target proteins. Many proteins exist in inactive and active forms, which differ by their phosphorylation state. Thus protein kinase activity can regulate the activity of other proteins. The phosphorylation is readily reversible by the action of protein phosphatase. The protein kinase of *Plodia interpunctella* GV (PiGV) has been found to phosphorylate the PiGV basic protein enabling the release of viral DNA from the nucleocapsids at the beginning of the infection process (Wilson and Consigli, 1985a,b; Funk and Consigli, 1993). All protein kinase catalytic domains have been found to contain 11 subdomains (Hanks *et al.*, 1988). These conserved subdomains are interspersed with non-conserved areas and contain a number of conserved amino acids. These areas are probably important in substrate binding and catalytic function; the non-conserved areas probably loop out to bring the catalytic subdomains together, this is typical for globular proteins (Hanks *et al.*, 1988). The consensus motifs include motifs for serine/threonine protein kinases and for ATP binding (Hanks *et al.*, 1988). AoGV contains all eleven subdomains with nine matching the consensus exactly. Consensus motif I is known to be involved in ATP-binding. This region is variable in AoGV. However, variability has been shown in this region in PxGV (Hashimoto *et al.*, in press) and in protein kinases from other organisms (Russell and Nurse, 1987; Saxena *et al.*, 1987; Lungdren *et al.*, 1991; Baylis *et al.*, 1993). Consensus motif X is not present in AoGV, but is also highly variable in other baculovirus proteins. It was also found to be the least conserved subdomain of the eleven (Hanks *et al.*, 1988). An alignment of baculovirus protein kinases is shown in Figure 4.22.

To confirm the activity of the protein, an assay of the protein, produced by *in vitro* translation of the gene would be required, as was performed with LdMNPV and AcMNPV protein kinases (Bischoff and Slavicek, 1994; Reilly and Guarino, 1994). The amino acid identity to baculovirus homologues is shown in Table 4.17. Phylogenetic analysis of protein kinase genes is shown in Figure 4.23. From these analyses it is clear that there are two subgroups, the GVs and the NPVs. Within the GVs the tortricid-infecting GVs appear to group and the noctuid and yponomeutid-

Figure 4.22

Multiple alignment of the amino acid sequence of baculovirus protein kinase proteins. Common amino acids are shaded pink.

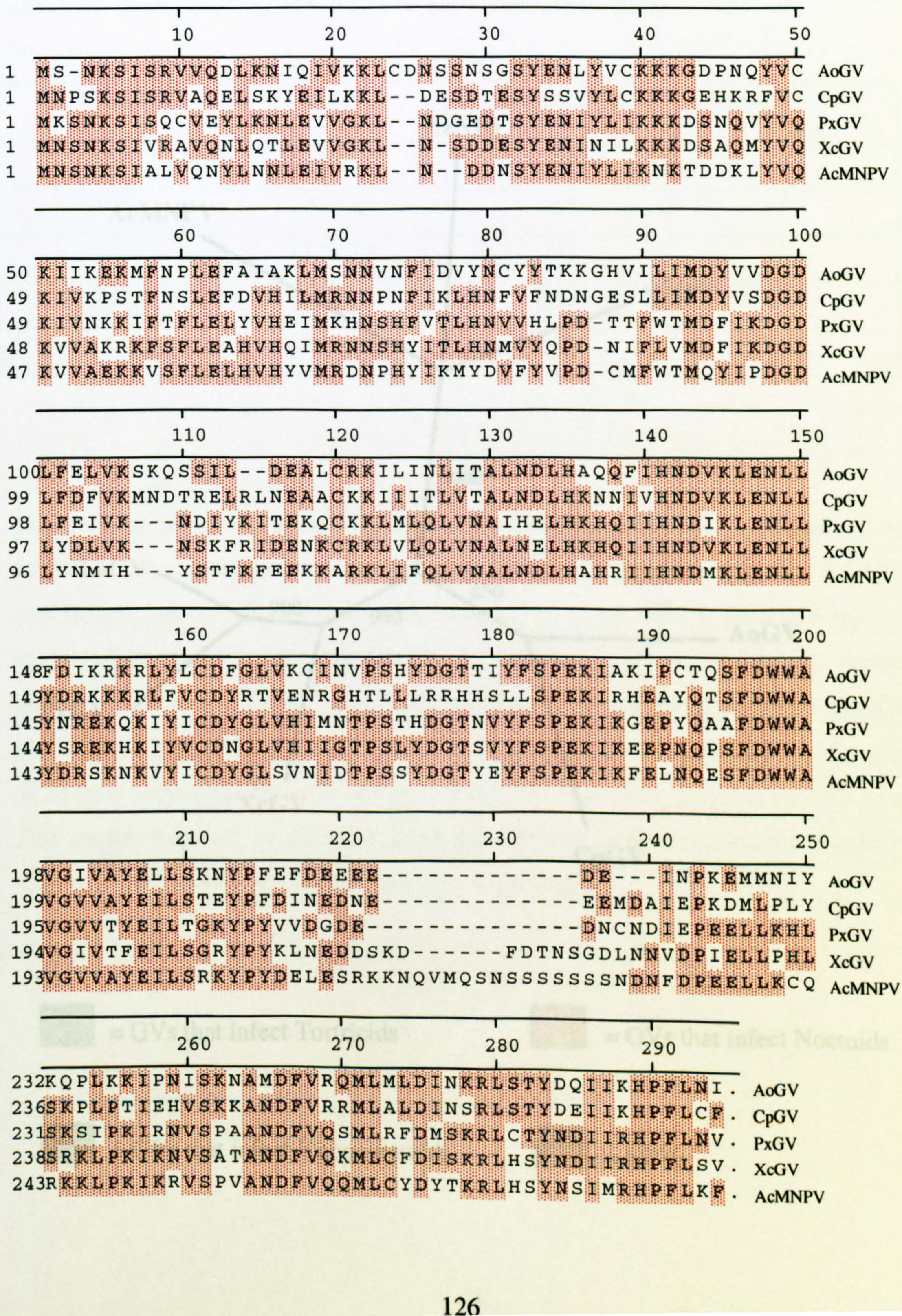
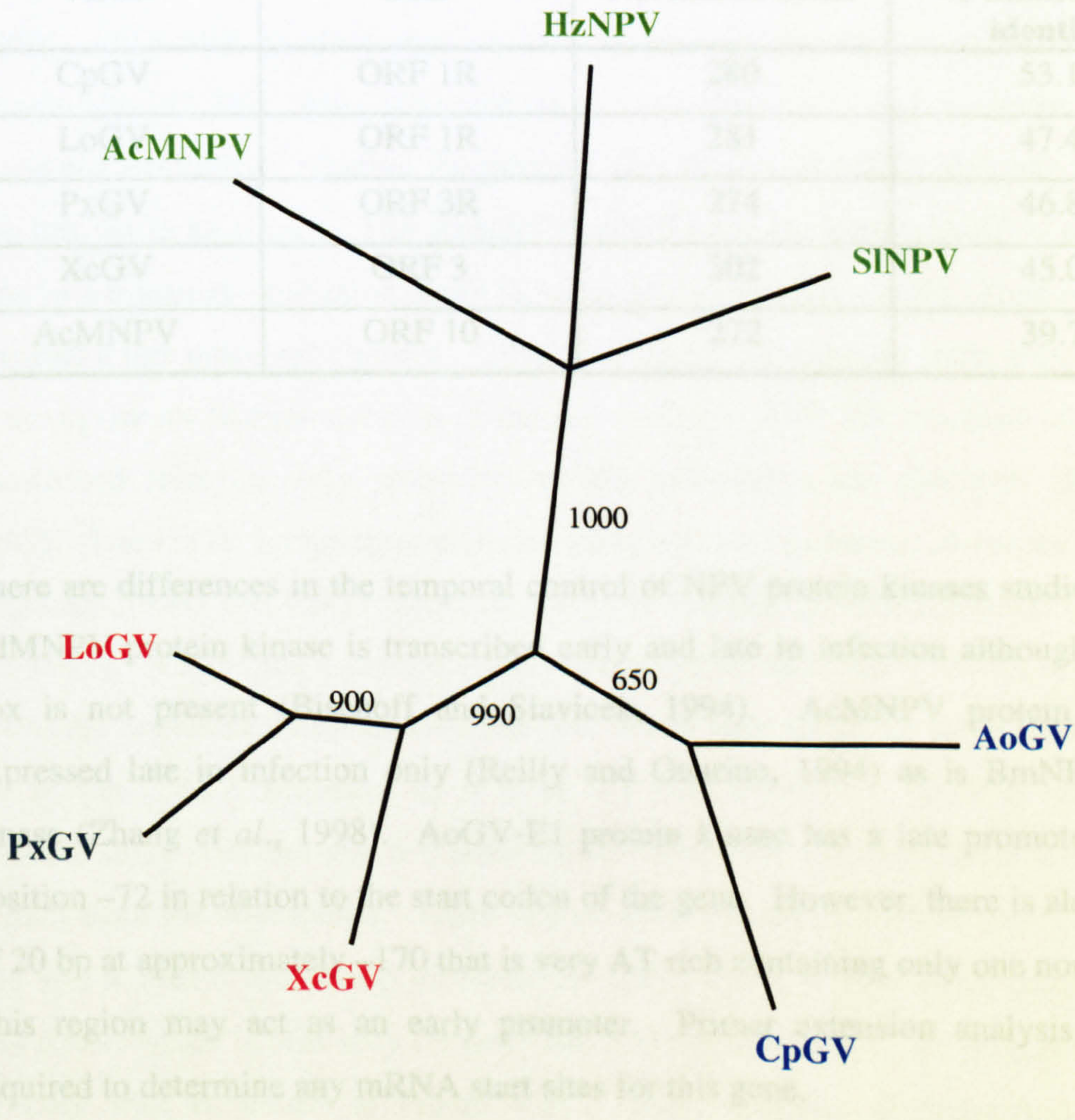


Figure 4.23

Phylogenetic analysis of baculovirus protein kinase amino acid sequences (section 2.12). Numbers indicate support, per 1000 bootstrap replications, for each internal branch with which they are juxtaposed.



= GVs that infect Tortricids

= GVs that infect Noctuids

= GVs that infect Yponomeutids

= NPVs

infecting GVs appear to group in this study. However, more sequences need to be compared before firm conclusions can be drawn.

Table 4.17

The size of granulovirus protein kinase proteins and AcMNPV protein kinase protein and the % amino acid identity to AoGV-E1 protein kinase protein.

Virus	ORF	No. amino acids	% amino acid identity
CpGV	ORF 1R	280	53.1
LoGV	ORF 1R	281	47.4
PxGV	ORF 3R	274	46.8
XcGV	ORF 3	302	45.0
AcMNPV	ORF 10	272	39.7

There are differences in the temporal control of NPV protein kinases studied to date. LdMNPV protein kinase is transcribed early and late in infection although a TATA box is not present (Bischoff and Slavicek, 1994). AcMNPV protein kinase is expressed late in infection only (Reilly and Guarino, 1994) as is BmNPV protein kinase (Zhang *et al.*, 1998). AoGV-E1 protein kinase has a late promoter motif at position –72 in relation to the start codon of the gene. However, there is also a region of 20 bp at approximately –170 that is very AT rich containing only one non-AT base. This region may act as an early promoter. Primer extension analysis would be required to determine any mRNA start sites for this gene.

4.2.13.4 ME53 (ORF 99R)

The function of this gene is unknown but similar genes are present in other baculoviruses, Table 4.17. Sequence analysis of the AoGV-E1 protein suggested two zinc finger motifs, one at the N-terminus C-X_2-C-X_{33}-C-X_2-C and one at the C-terminus C-X_2-C-X_{14}-C-X_2-C, Figure 4.24. These zinc fingers are also conserved in other granulovirus homologues, Figure 4.24. The presence of zinc fingers consisting only of clustered cysteine residues, as opposed to cysteine and histidine residues, has been observed in the steroid hormone nuclear receptor family of proteins and is thought to be essential for sequence-specific recognition of DNA (Freedman *et al.*, 1988). ORF 99R product showed low amino acid identity to AcMNPV ME53 homologues (22.3%). Also, the presence of a cysteine zinc finger indicates a possible functional similarity. Whether the putative zinc fingers in ME53 play any functional role has yet to be shown. The AcMNPV ME53 gene has been identified as an early gene in a transient expression assay (Knebel-Mörsdorf *et al.*, 1996). However, it also contains a late promoter element. Other GV ME53 homologues show little similarity in the upstream promoter region of the gene. CfGV ORF 891 has been shown to be transcribed from an early promoter but also contains a late promoter (Bah *et al.*, 1999). The ClGV homologue contains early and late promoter elements (Jehle and Backhaus 1994) and CpGV contains early but not late promoter elements (Crook *et al.*, 1997). AoGV has a potential early transcription start site (CACT) at –40 and a TAATAT sequence 29 bp upstream at –69, which may act as the promoter rather than the normal TATA sequence as it is an AT-rich sequence. There is also another possible early start site and promoter further upstream, although these are unusually distant from the start codon. There is a possible transcription start site (CATA) at –135 and a TATA box 23 bp upstream at –158. There is also a late promoter motif (ATAAG) which is also very far from the start codon at –184. Phylogenetic analysis of ORF 99R is shown in Figure 4.25. These analyses distinguished two basic subgroups. One subgroup comprised of ME53 homologues from noctuid and yponomeutid infecting GVs and the other from GVs that infect tortricids.

Figure 4.24
Alignment of amino acid sequences of baculovirus ME53 homologues. Common amino acids are shaded pink. Cysteine residues that form zinc fingers are boxed.

Figure 4.25

Phylogenetic analysis of GV ME53 homologue protein sequences (section 2.12). Numbers indicate support, per 1000 bootstrap replications, for each internal branch with which they are juxtaposed.

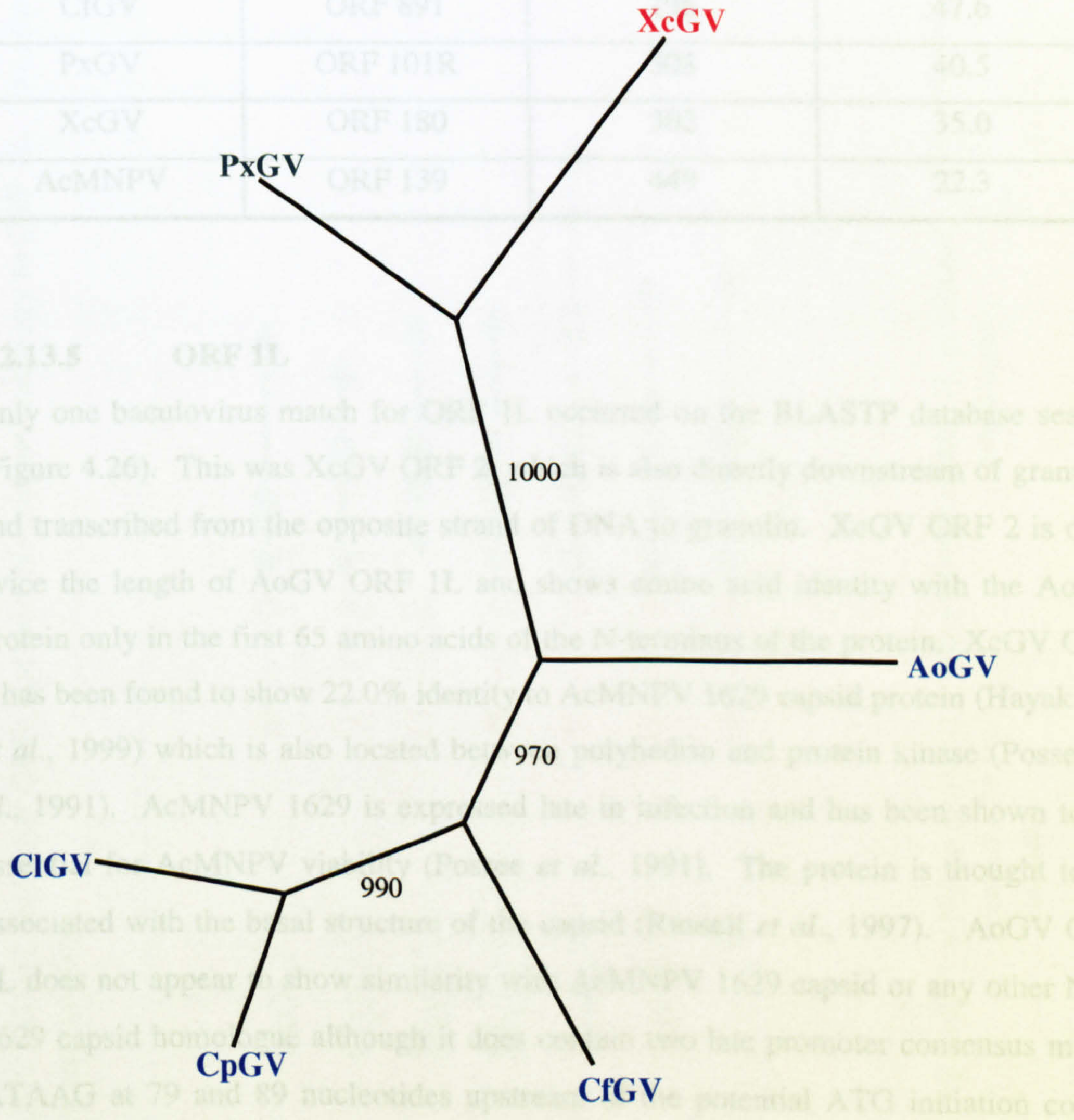


Table 4.18

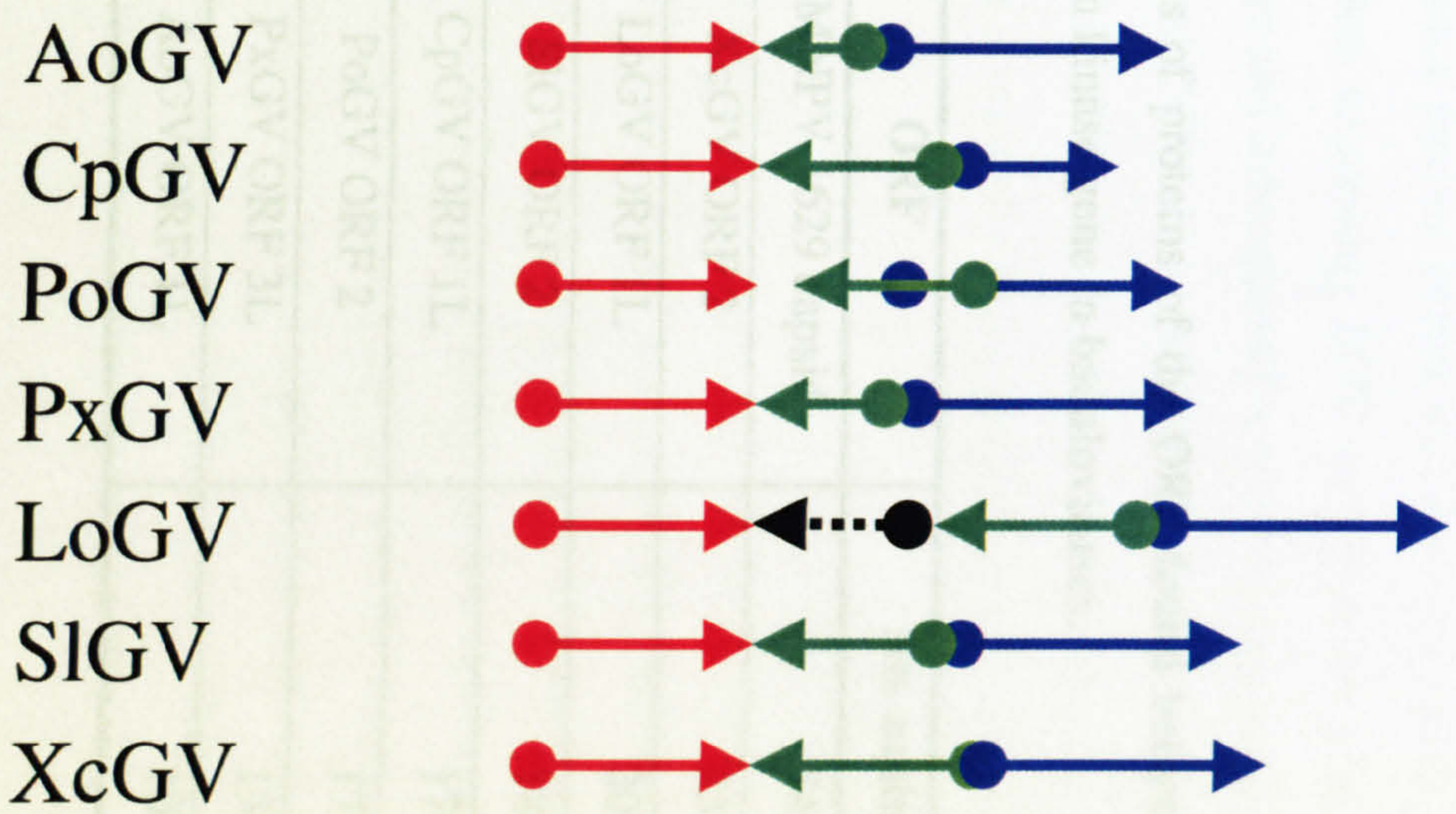
The lengths of AcMNPV ME53 protein and granulovirus ME53 homologue proteins and the % amino acid identity to the product of AoGV ORF 99R.

Virus	ORF	No. amino acids	% amino acid identity
ClGV	ORF 909	303	47.8
CpGV	ORF 124R	306	47.7
CfGV	ORF 891	296	47.6
PxGV	ORF 101R	308	40.5
XcGV	ORF 180	302	35.0
AcMNPV	ORF 139	449	22.3

4.2.13.5 ORF 1L

Only one baculovirus match for ORF 1L occurred on the BLASTP database search (Figure 4.26). This was XcGV ORF 2, which is also directly downstream of granulin and transcribed from the opposite strand of DNA to granulin. XcGV ORF 2 is over twice the length of AoGV ORF 1L and shows amino acid identity with the AoGV protein only in the first 65 amino acids of the N-terminus of the protein. XcGV ORF 2 has been found to show 22.0% identity to AcMNPV 1629 capsid protein (Hayakawa *et al.*, 1999) which is also located between polyhedrin and protein kinase (Possee *et al.*, 1991). AcMNPV 1629 is expressed late in infection and has been shown to be essential for AcMNPV viability (Possee *et al.*, 1991). The protein is thought to be associated with the basal structure of the capsid (Russell *et al.*, 1997). AoGV ORF 1L does not appear to show similarity with AcMNPV 1629 capsid or any other NPV 1629 capsid homologue although it does contain two late promoter consensus motifs ATAAG at 79 and 89 nucleotides upstream of the potential ATG initiation codon. Other ORFs from GVs that are immediately downstream from granulin differ in length from 106 amino acids in AoGV to 231 in XcGV (Table 4.18) but all show identity in the first 65 amino acids of the amino terminal region of the protein. All of these ORFs are transcribed on the opposite strand to granulin and most either terminate very close to the end of the granulin sequence or overlap slightly (Figure 4.26). However, LoGV ORF 1L terminates 450 bp downstream of the end of the

Figure 4.26
ORF arrangement around AoGV ORF 1L and other ORFs that share N-terminal homology to ORF 1L. The arrows represent the ORFs and the direction of transcription.



= Granulin

= No baculovirus homologue on database

= ORF 1L positional homologue

= Protein kinase

granulin sequence and there is an additional putative ORF between it and granulin which does not have any similarity to baculovirus ORFs on the database (I. R. L. Smith, personal communication). There is also a gap of about 100 bp between the 3' end of PoGV granulin gene and the 3' end of PoGV ORF 2. All of the ORFs overlap at their 5' end with the 5' end of the protein kinase gene, mostly by 16 or 19 bp. This could explain the amino acid similarity between the ORFs at the 5' end. However, the 5' end of PoGV ORF 2 overlaps by 247 bp with the 5' end of the protein kinase gene and XcGV overlaps by 67 bp. Therefore, the similarity at the amino terminus is more likely to be a characteristic of the protein rather than due to the similarity of the 5' ends of the protein kinase genes.

Table 4.19 Lengths of proteins of the ORF found between the granulin/polyhedrin gene and the protein kinase gene in baculoviruses.

ORF	No. amino acids
AcMNPV 1629 capsid	543
XcGV ORF 2	231
LoGV ORF 1L	205
SlGV ORF 2	188
CpGV ORF 1L	174
PoGV ORF 2	173
PxGV ORF 3L	131
AoGV ORF 1L	106

4.2.13.6 Ecdysteroid UDP-glucosyl transferase (EGT)

The *egt* genes of baculoviruses encode ecdysteroid UDP-glucosyl transferase (EGT) which belongs to the UDP-glucosyl transferase superfamily. These transferases all conjugate small lipophilic compounds with various sugars (O'Reilly and Miller, 1989). EGT is secreted into the insects haemolymph and it is here that it catalyses the conjugation of ecdysteroids, the insect moulting hormones, with the sugar moiety donated from UDP-glucose or UDP-galactose (O'Reilly *et al.*, 1992). This inactivates the moulting hormone and thereby prevents moulting and pupation (O'Reilly and Miller, 1989, 1991). Therefore, larvae usually die in the instar in which they were infected. Uninfected larvae cease feeding before and during a moult. In contrast, larvae infected with a wild-type virus expressing EGT do not moult and therefore continue to feed, thereby getting larger and subsequently increasing the yield of virus. Infection of larvae by a recombinant AcMNPV virus lacking the gene resulted in reduced feeding and an increased speed of kill (O'Reilly and Miller 1991; Flipsen *et al.*, 1995). Therefore the deletion of this gene is currently being studied as a strategy for improving baculoviruses as biocontrol agents.

The *egt* gene has been identified in many baculoviruses and appears to be a common gene. The only genome so far that has not been found to contain an *egt* gene is that of XcGV (Hayakawa et *al.*, 1999). XcGV is a slow-killing virus and does not kill within the instar it infects so the absence of an *egt* gene is not surprising. AoGV is also a slow killing virus, infected larvae always die late in final instar, irrespective of which instar they were infected. Therefore it was expected that AoGV would not have an *egt* gene or at least would not have one which was functional. However, an *egt* homologue was found upstream of ORF 99R in the same location as CpGV *egt*.

The AoGV EGT protein has been compared to other members of the EGT family, the amino acid identity being given in Table 4.20 and the alignment shown in Figure 4.27. Phylogenetic analysis has also been performed and is shown in Figure 4.28. From these analyses it is clear that the GVs group. However, SlMNPV appears to group weakly with the GVs and the NPVs have two groupings with HzNPV and LdMNPV belonging to neither. In this case the tortricid infecting CpGV groups more strongly with the noctuid infecting LoGV than the tortricid infecting AoGV.

Figure 4.27
Alignment of the amino acid sequence of 4 baculovirus EGT proteins. The conserved regions are indicated with roman numerals (I-X) and green lines. The seven absolutely conserved amino acids among all the UDP-glucosyl transferases are indicated by asterisks (*). The signal peptide is boxed in blue. Common amino acids are shaded pink.

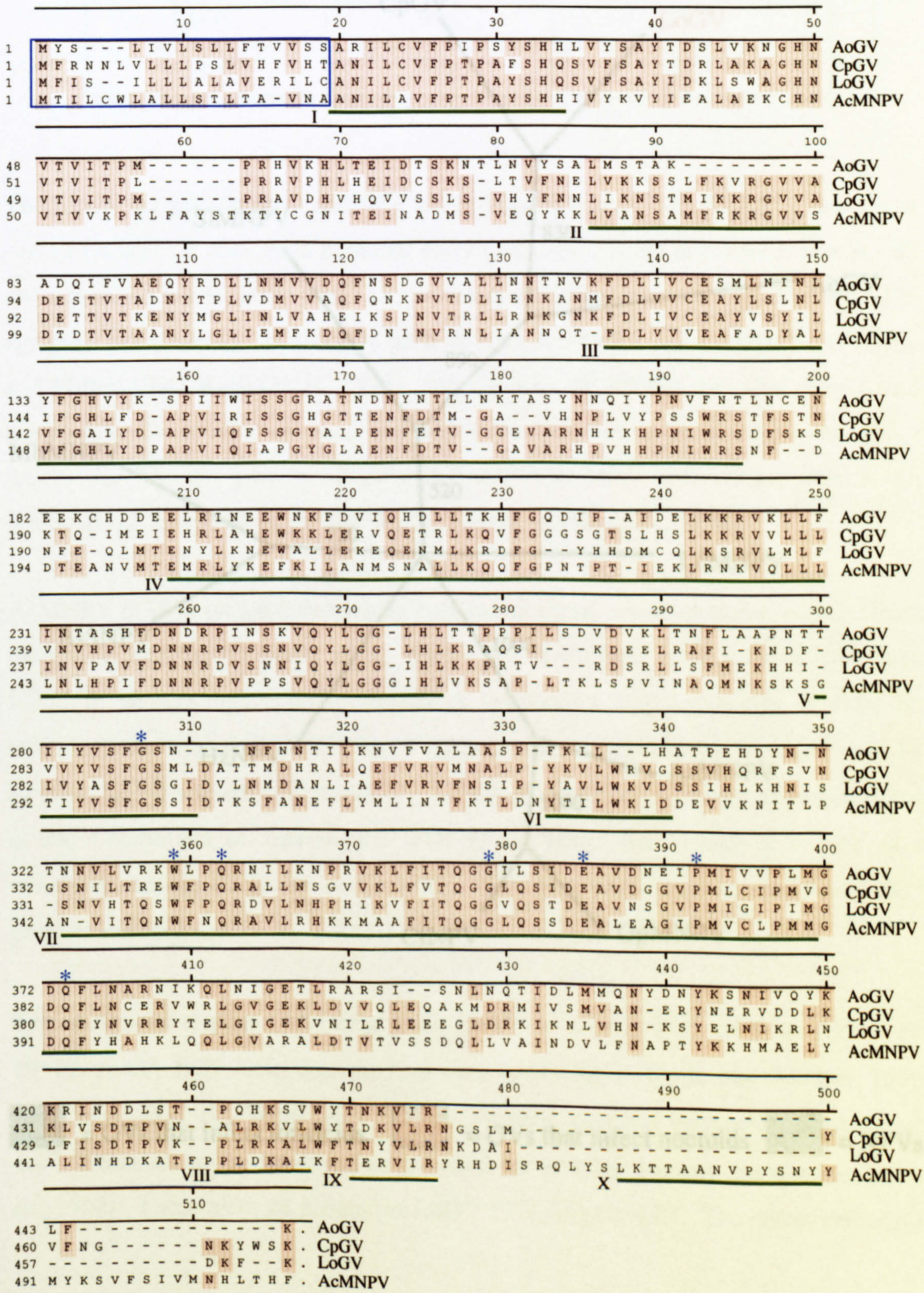
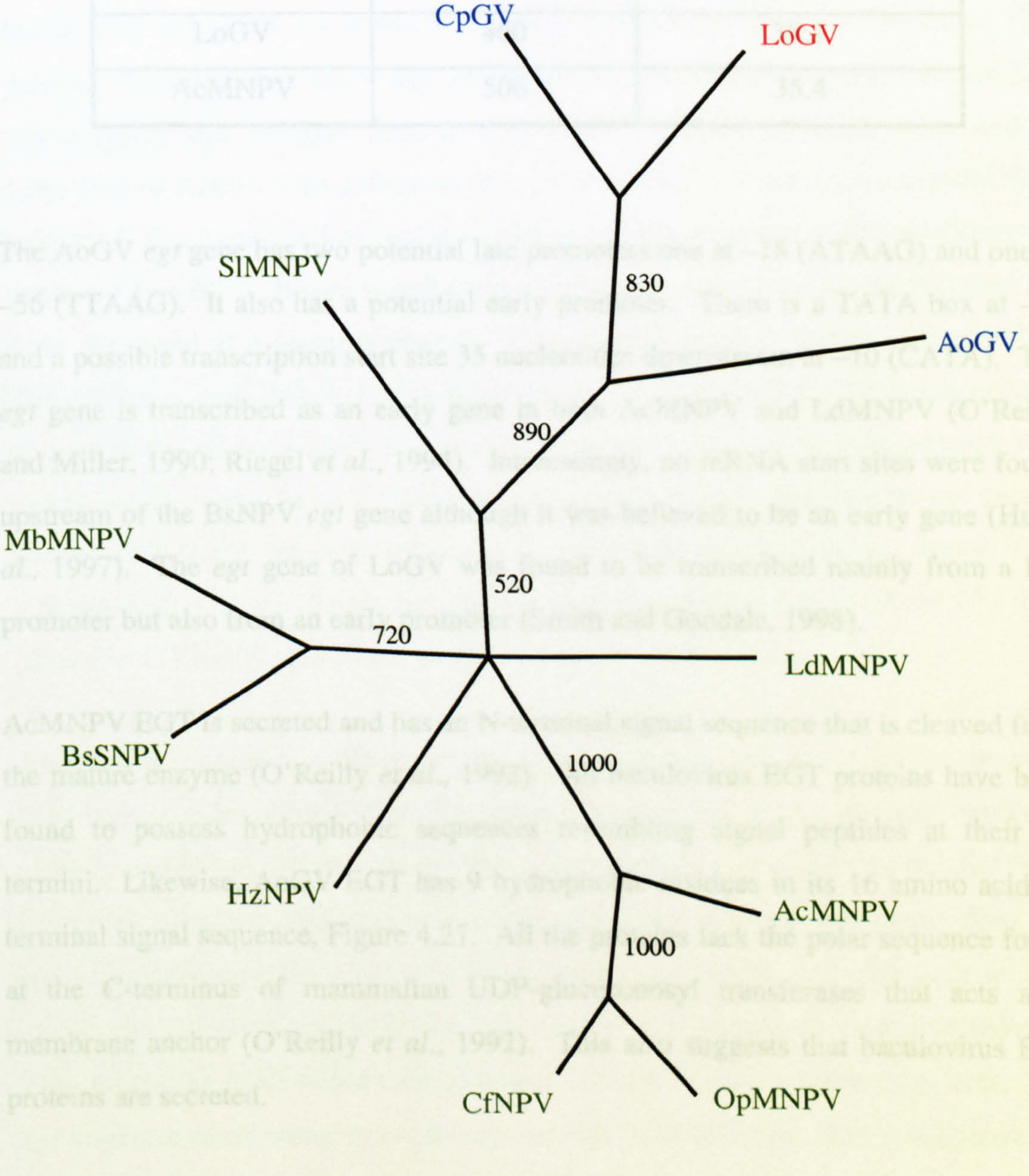


Figure 4.28

Phylogenetic analysis of baculovirus EGT protein sequences (section 2.12). Numbers indicate support, per 1000 bootstrap replications, for each internal branch with which they are juxtaposed.



= GVs that infect tortricids = GVs that infect noctuids = NPVs

Table 4.20

The lengths of granulovirus EGT homologue proteins and AcMNPV EGT protein and the % amino acid identity to AoGV EGT.

Virus	No. amino acids	% amino acid identity
CpGV	484	41.7
LoGV	460	38.3
AcMNPV	506	35.4

The AoGV *egt* gene has two potential late promoters one at –18 (ATAAG) and one at –56 (TTAAG). It also has a potential early promoter. There is a TATA box at –45 and a possible transcription start site 35 nucleotides downstream at –10 (CATA). The *egt* gene is transcribed as an early gene in both AcMNPV and LdMNPV (O'Reilly and Miller, 1990; Riegel *et al.*, 1994). Interestingly, no mRNA start sites were found upstream of the BsNPV *egt* gene although it was believed to be an early gene (Hu *et al.*, 1997). The *egt* gene of LoGV was found to be transcribed mainly from a late promoter but also from an early promoter (Smith and Goodale, 1998).

AcMNPV EGT is secreted and has an N-terminal signal sequence that is cleaved from the mature enzyme (O'Reilly *et al.*, 1992). All baculovirus EGT proteins have been found to possess hydrophobic sequences resembling signal peptides at their N-termini. Likewise, AoGV EGT has 9 hydrophobic residues in its 16 amino acid N-terminal signal sequence, Figure 4.27. All the proteins lack the polar sequence found at the C-terminus of mammalian UDP-glucuronosyl transferases that acts as a membrane anchor (O'Reilly *et al.*, 1992). This also suggests that baculovirus EGT proteins are secreted.

Ten conserved regions (I-X) have been identified among EGT proteins (Hu *et al.*, 1997). AoGV EGT lacks domain X as does LoGV EGT (Smith and Goodale, 1998) and CpGV EGT (D. Winstanley and D. O'Reilly, personal communication), Figure 4.27. There are seven conserved amino acids for all UDP-glucosyl transferases (Hu *et al.*, 1997). These were all present in AoGV EGT, Figure 4.27. The conserved region

II has nine amino acids absent in AoGV, four of which are conserved in all other baculovirus EGT proteins sequenced to date. The significance of this is not clear.

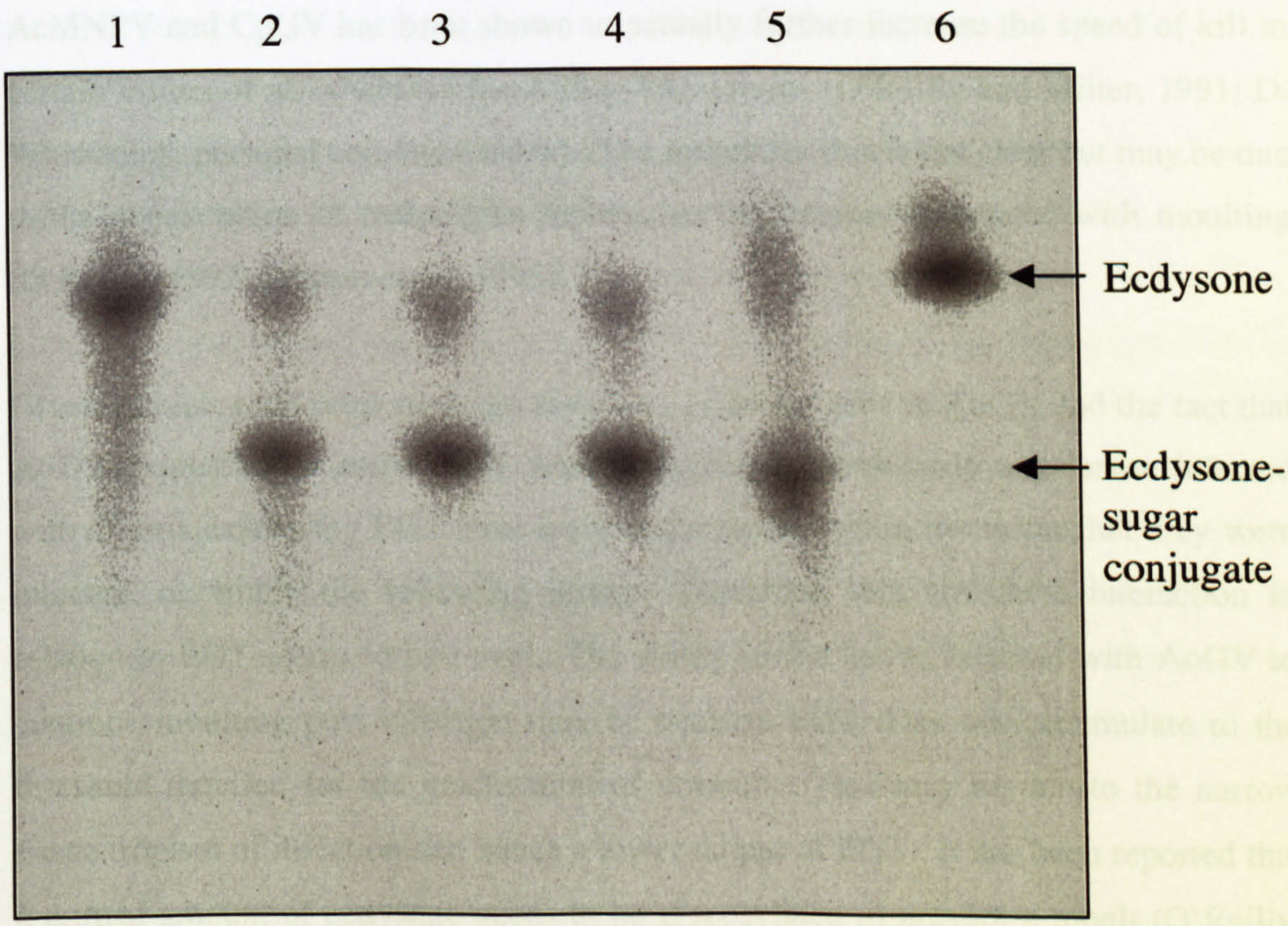
4.2.13.7 Assay of EGT activity to determine whether AoGV produces a functional EGT protein

It was now known that AoGV contained a gene with high similarity to other baculovirus *egt* genes but it was not known if AoGV produced a functional EGT protein. Therefore this assay was performed to address this question (section 2.9). Haemolymph was collected from *A. orana* larvae infected as fourth instar with an LD_{95} dose of AoGV. The collection time points were 5, 10 and 15 d p.i. AcMNPV-infected *S. frugiperda* cell extract was used as a positive control as this was known to contain active EGT. Haemolymph from uninfected fourth instar larvae collected at 5 d p.i. was used as a negative control. The autoradiograph is shown in Figure 4.29. The results proved that AoGV was producing an active EGT. No conjugation of glucose to the ^{3}H-ecdysone was observed in the negative control (haemolymph from uninfected *A. orana* larvae). Conjugation was observed in the case of the positive control (AcMNPV infected cell extract) and haemolymph from *A. orana* larvae infected with AoGV. Ecdysone was the preferred ecdysteroid for AcMNPV EGT (Evans and O'Reilly, 1998) and was therefore used in this assay.

Although it is extremely likely that the *egt* gene sequenced does code for the active EGT being produced by AoGV, this cannot be confirmed until a transient expression assay is performed, (Clarke *et al.*,1996). This would involve cloning the AoGV *egt* gene plus a few hundred base pairs either side into a plasmid such as pBSK+. This would then be co-transfected into an AcMNPV permissive cell line such as Sf21, along with infection by vEGTDEL, an AcMNPV recombinant virus that lacks *egt*. The cell culture fluid would then be assayed for EGT activity a few days later. If the *egt* sequence could complement the *egt*-deleted AcMNPV then EGT would have been produced and this would confirm that the AoGV *egt* sequence does encode an active EGT.

Figure 4.29
Ecdysteroid UDP-glucosyl transferase assay using the substrate ^{3}H-ecdysone. Haemolymph from uninfected *A. orana* (negative control) and fourth instar *A. orana* infected with an LD_{95} dose at various d p.i. and AcMNPV infected cell extract (positive control).

1 = negative control
2 = Haemolymph 5 d p.i.
3 = Haemolymph 10 d p.i.
4 = Haemolymph 15 d p.i.
5 = Positive control
6 = ^{3}H-ecdysone



4.3 Discussion

4.3.1 Bioassays and EM studies

From the AoGV bioassay results it was clear, that regardless of the instar in which the larvae were infected, they did not die, until on average 25 days after reaching fifth instar. Furthermore, external symptoms of infection were not obvious before the fifth instar. This suggests that there could be a 'switch' that occurs during the fifth instar, which allows the virus infection to proceed. The lack of an *egt* gene in slow GVs e.g. XcGV seemed likely to be responsible for the ability of infected larvae to undergo consecutive moults until the final instar. The infection of predominantly the fat body in these infections is also thought to contribute to the slow infection, as other vital organs are not infected (Federici, 1997). However, the removal of the *egt* gene in AcMNPV and CpGV has been shown to actually further increase the speed of kill in certain instars of an otherwise fast-killing baculovirus (O'Reilly and Miller, 1991; D. Winstanley, personal communication). The reason for this is not clear but may be due to the degeneration of malphigian tubules, or the stresses associated with moulting (O'Reilly, 1997; Flipsen *et al.*, 1995).

Given its biological properties, the discovery of an *egt* gene in AoGV and the fact that AoGV expressed an active EGT were unexpected. Previously any larvae infected with a virus expressing EGT have been found to die within the instar that they were infected, or within the following instar. Therefore, this virus/host interaction in relation to EGT seems to be novel. The ability of the larvae infected with AoGV to continue moulting post infection may be because EGT does not accumulate to the threshold required for the inactivation of a moult. This may be due to the narrow tissue tropism of infection and hence a lower output of EGT. It has been reported that a critical amount of ecdysone needs to be glucosylated to prevent a moult (O'Reilly, 1997). Very few symptoms were observed in the infected larvae until the fifth instar, suggesting that the amount of viral replication and therefore the EGT production is low. Once the larvae had reached fifth instar, the infection appeared to proceed rapidly and external symptoms were observed. The fat body appeared white and hypertrophied within a few days. The infected larvae failed to pupate when the control larvae pupated and therefore the EGT levels must have been high enough by then to prevent the moult.

The larvae remained in a prolonged fifth instar for several days, during which time many discharged large amounts of virus. This may be a mechanism of disseminating virus to infect further larvae before they die. Most larvae died as larvae/pupae intermediates. Larvae that did not reach this stage before death were usually those that had discharged so much liquid that they could not survive. The fact that several days before death the larvae actually tried to pupate, be it on average 20 days after the controls, suggested hormonal disruption. A similar symptom was observed with larvae exposed to fenoxycarb, a juvenile hormone (JH) analogue (Cross, 1997). JH levels normally remain high throughout larval development but shortly before pupation they drop dramatically due to the accumulation of juvenile hormone esterase (JHE) (Black *et al.*, 1997). Fenoxycarb persists at a high level, so the larvae only partially pupate resulting in abnormalities, which are lethal (Cross, 1997). Therefore, it seems possible that AoGV-infected larvae have either an increase in JH or a decrease in JHE several days before they die. EGT levels may also drop, allowing this partial pupation to occur. The EGT assay will be carried out with larvae that have been infected as neonates, at different time points. This would clarify whether EGT is present throughout infection or just towards fifth instar. The haemolymph from partial pupae should also be assayed to see whether EGT levels decrease. It would also be very interesting to assay the levels of juvenile hormone esterase (JHE) to determine whether this enzyme contributes to the development of larvae/pupae intermediates. The apparent lack of these abnormal larvae/pupae intermediates in GV-infected smaller tea tortrix larvae is very interesting. JHE assays could also be performed on these larvae infected with AoGV to determine whether there are any hormonal differences during infection between the two larval species.

Because AoGV-infected larvae die as partial pupae, they do not lyse, therefore, virus is not released into the environment at this stage. Instead, the larvae simply dry out. It is interesting to note that homologues of chitinase and cathepsin could not be detected in the AoGV genome by hybridisation. These genes are involved in the breakdown of the cuticle in larvae infected with other baculoviruses, which makes them fragile (Ohkawa *et al.*, 1994; Slack *et al.*, 1995; Hawtin *et al.*, 1997). The insects then 'melt', releasing virus into the environment. Therefore, in the case of

AoGV-infected *A. orana* larvae, the discharge of virus in fluid exudate prior to death may be the predominant method of transmitting the virus.

The times to death for AoGV-infected larvae ranged over a long period in each experiment. In one experiment, a larva infected as a neonate survived much longer than the other infected larvae, dying at 112 days. Considering that the healthy controls all pupated by the age of 22 days and became adults by 28 days, this was quite impressive. This larva did not exude virus or become a larva/pupa intermediate and remained quite small throughout infection, although was still active and feeding. The OBs recovered from this larva were fed to further neonates to see if the altered phenotype persisted. However, the larvae did not take any longer to die than in normal infections. Therefore this unusual result is unexplained.

The EM studies showed that the fat body was highly infected. A larva that was infected with AoGV at fourth instar contained fat body cells full of OBs by 4.5 d p.i. At this stage the larva did not show any external signs of infection. Therefore, this could represent an early phase in the infection process. However, it is possible that only some of the fat body cells are heavily infected throughout the course of infection. It is also possible that the OB-containing fat body cells do not lyse until the final instar so the haemolymph remains clear. It is difficult at this stage to say where the infection block prior to fifth instar occurs, or even if there is one. It appears from the non-milky appearance of the larvae that OBs are not produced until fifth instar. However, they may be produced in a small proportion of fat body cells before fifth instar.

The EM studies also indicated that AoGV OBs may be present in midgut cells, this was unexpected as baculovirus OBs are not usually produced in midgut cells. The only GV that results in this tissue infection is *Harrisina brillians* GV (HbGV) (Smith *et al.*, 1956). HbGV infects only midgut epithelium cells and results in diarrhoea, which will eventually kill the larvae (Smith *et al.*, 1956; Crook, 1991). As with AoGV infection, the diarrhoea and frass of these larvae are the source of infection for further larvae, as the larvae do not lyse. Although AoGV OBs were only seen in a few midgut cells in the experiments described here, it is possible that these cells could be the origin of the viral discharge. One larva used for the EM studies 13.5 d p.i had

already discharged virus. The midgut of this larva appeared grey and tattered suggesting that a midgut infection of AoGV may induce diarrhoea in *A. orana* larvae. A more extensive EM study will need to be performed on larvae infected as neonates to look at a variety of tissues at different time points to confirm this possibility.

There may be possible synergism between AoGV and AoNPV infections *in vivo*. The original infected larvae from which the AoGV stock was derived were found to have equimolar amounts of NPV and GV DNA. Larvae infected with this mixture of NPV and GV died faster than larvae infected with the GV alone but slower than larvae infected with the subsequently purified NPV. The larvae were large when they died from a mixed GV/NPV infection but did not partially pupate. In NPV infections alone, the larvae died very small and the yield of virus was low. In GV infected larvae the virus took much longer to kill. In a mixed infection it appears that the yield of NPV was higher than when the larvae were infected predominantly with the NPV possibly because the infection takes longer. It may be possible that AoGV contains an enhancin gene. Enhancins (synergistic factor or viral enhancing factor) are proteins produced by some baculoviruses that can dramatically enhance the oral infectivity of other baculoviruses (Derksen and Granados, 1988; Goto, 1990; Hayakawa *et al.*, 2000). Enhancin is a metalloprotease which affects virus infection by altering the structural integrity of the peritrophic membrane which is a protective lining secreted by the midgut (Lepore *et al.*, 1996; Wang *et al.*, 1994). Genes encoding enhancins have been identified from several GVs including XcGV, which has four enhancin genes (Hayakawa *et al.*, 1999), *Pseudaletia unipuncta* GV (PuGV) and *Helicoverpa armigera* GV (HaGV) (Roelvink *et al.*, 1995) and TnGV (Hashimoto *et al.*, 1991). In addition two enhancin genes have been identified in LdMNPV (Kuzio *et al.*, 1999). If AoGV does encode an enhancin then the entry of NPV through the peritrophic membrane will be enhanced and it will be an advantage for the NPV to coinfect with the GV. However, hybridisation was not detected using TnGV enhancin gene as a probe against AoGV DNA (data not shown). The advantage of the GV associating with the NPV may be that it kills faster and can therefore infect larvae of the same generation. Usually the GV is not released until the current generation of larvae has pupated and so it is the next generation that is targeted. The NPV is proving very difficult to separate completely from the GV, possibly due to its dependence on the GV for its efficient replication. However, the

presence of large masses of GVs within a matrix of some kind, may be the reason why the physical separation of NPV from GV using gradients has been unsuccessful.

4.3.2 Mapping and sequencing

The mapping of the AoGV-E genome was a prerequisite to any further genome studies. The mapping allowed the collinearity and similarity studies of the different GV genomes to be carried out (chapter 3). The *Eco*RI map will allow a more precise analysis to be performed and also provides the basis for a complete sequencing project, of which the sequencing of the granulin region was the first phase. The complete genome should aid in the search for genes that govern tissue tropism and speed of kill.

The AoGV genome is relatively small and showed moderate similarity to the fast GV CpGV, based on hybridisation studies (chapter 2). The AoGV genes sequenced so far showed most similarity to CpGV genes, although to date few GV genes have been sequenced. However, this information is very useful since the sequencing of the CpGV genome is near completion and a direct comparison of the genes of CpGV and AoGV will then be possible. Another slow-killing virus, XcGV, has recently been sequenced and the fast-killing PxGV sequence will soon be available (Hayakawa *et al.*, 1999; Hashimoto *et al.*, in press). With more GV sequences available, the similarities and differences between them will become more apparent.

The gene organisation in the granulin region of AoGV was similar to other tortricid-infecting viruses (CpGV, ClGV, and CfGV). In contrast, the organisation of this region in noctuid-infecting viruses (LoGV, SlGV, XcGV, and TnGV) and GVs that infect other families showed more diversity. First, the noctuid viruses did not have an ME53 homologue directly upstream of the granulin gene. In XcGV, it was displaced one ORF upstream (Hayakawa *et al.*, 1999). Whereas in PxGV it was displaced two ORFs upstream (Hashimoto *et al.*, 1999). A ME53 homologue was not identified within the granulin area of LoGV. Second, PxGV was found to have a possible *p10* homologue within the granulin-containing region (Hashimoto *et al.*, 1999) whereas no counterpart was identified here for AoGV. A *p10* homologue has also been identified in XcGV although the P10 proteins have very low amino acid identity to each other and to NPV P10 proteins (Hayakawa *et al.*, 1999). Third, the *egt* gene which is the

second reading frame upstream of the granulin gene in AoGV and other tortricid-infecting GVs lies 8 kbp upstream of granulin in LoGV. LoGV also contained an ORF with unknown function and an IAP homologue within the granulin region. The current gene arrangement within the granulin region of *Phthorimaea opercullella* GV (PoGV) which infects larvae from the family Gelechiidae is in close relation to the tortricids (Taha, *et al.*, 1999). Also, from the phylogenetic analysis of the granulin proteins, PoGV groups with the tortricid-infecting GVs.

The only gene found in a conserved position in relation to the granulin gene in all GVs so far sequenced except LoGV was the protein kinase gene, which is two ORFs downstream from the granulin gene. In LoGV the gene is three ORFs downstream due to the presence of an extra small ORF downstream of granulin. The protein kinase gene in AcMNPV, BmNPV and LdNPV is also two ORFs downstream of the polyhedrin (granulin-equivalent) gene and transcribed in the same direction (Ayres *et al.*, 1994; Gomi et al., 1999; Kuzio *et al.*, 1999). The ORF immediately downstream of granulin in XcGV, SlGV and PoGV has been found to share homology to ORF 1629 of AcMNPV which is immediately downstream of the polyhedrin gene (Hayakawa *et al.*, 1999; Taha *et al.*, 1999; Possee *et al.*, 1991). However, the ORF 1629 protein is much larger than those encoded by the GV-equivalent ORFs. No overall similarity with ORF 1629 was found in AoGV ORF 1L or the ORFs immediately downstream of granulin in LoGV, PxGV and CpGV. However, all of the GVs shared a similarity at the amino terminus of the protein. The function of this ORF is unknown at present but appears to be conserved as all GVs in this study contained this ORF.

The gene arrangement around the granulin area of GVs shows that conservation of certain genes has occurred. Usually the same set of genes is present with slight alterations or additions specific to each virus. The gene arrangement in tortricid-infecting GVs appears at present to be more conserved that that between noctuid-infecting GVs which appear to have more small ORFs that do not have similarity to other baculovirus ORFs. This analysis of gene arrangement among GVs supports my hypothesis that GVs are more closely related depending on the family of Lepidoptera they infect rather than their speed of kill or the tissues they infect within their target species.

CHAPTER 5

Studies to identify genes involved in host range determination in CpGV and to study its pathogenesis in various hosts.

5.1 Introduction

Baculoviruses have a relatively narrow host range, infecting usually only one host or a few closely related hosts (Gröner, 1986). NPVs generally have a broader host range than GVs. AcMNPV has the widest host range of any baculoviruses known to date, infecting at least 33 species of lepidopteran larvae in 10 families (Gröner, 1986). The granulovirus *Cydia pomonella* GV (CpGV) infects only a few closely related species including its native host *Cydia pomonella* and an alternative host *Cryptophlebia leucotreta*. However, *C. leucotreta* GV (ClGV) infects *C. leucotreta* but is not infectious to *C. pomonella*. In chapter 3, the genomes of ClGV and CpGV were shown to be collinear and to have relatively high similarity.

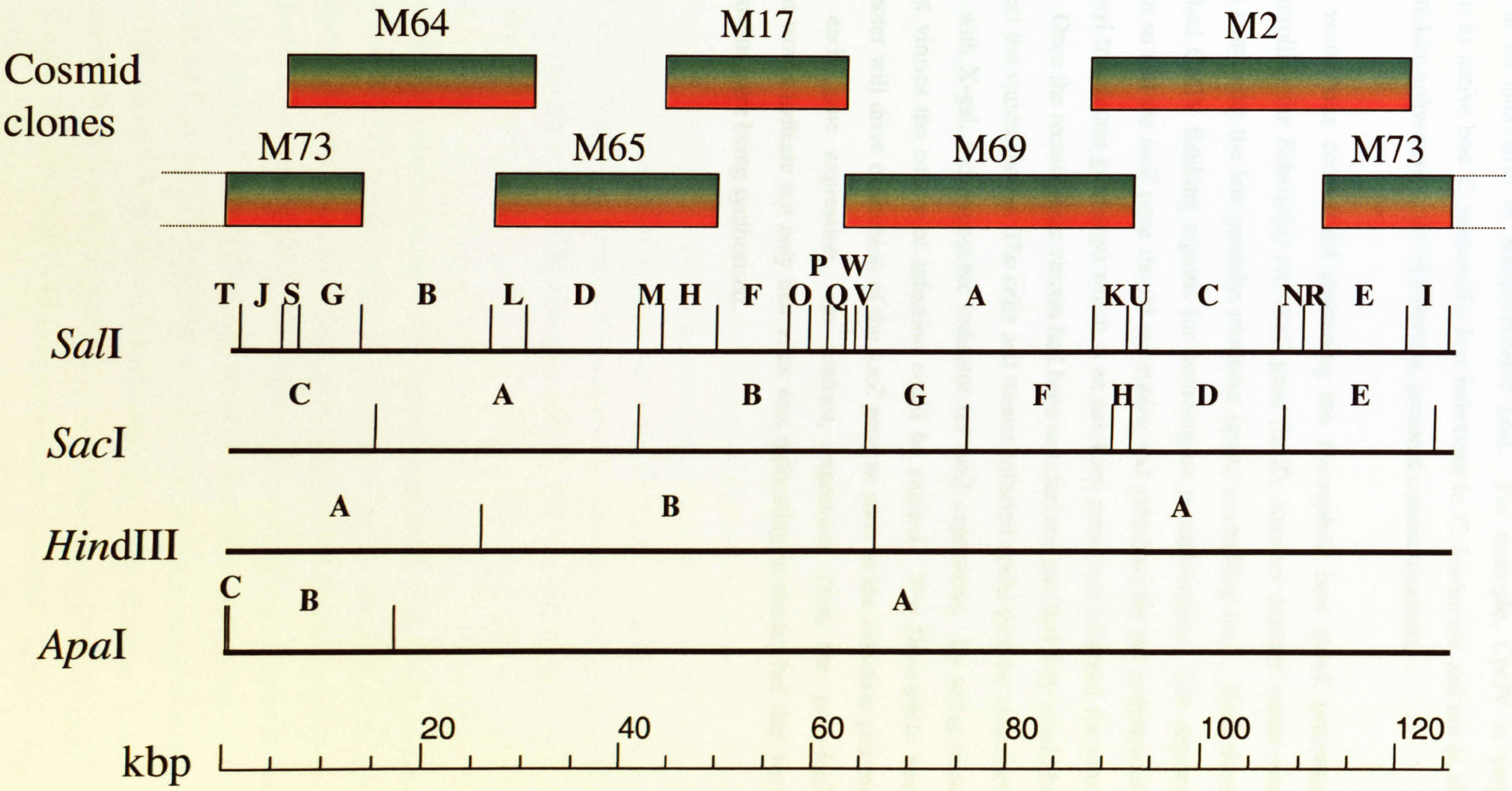
One of the studies undertaken in this chapter was to investigate the expansion of the ClGV host range to include *C. pomonella*. Previous studies on host range have involved NPVs with nonoverlapping host ranges but with high DNA similarity (Miller and Lu, 1997). CpGV and ClGV have overlapping host ranges since both viruses infect *C. leucotreta* and have relatively high DNA similarity. Therefore, these GVs seemed appropriate to use for a similar host range expansion study, particularly since a CpGV-permissive *C. pomonella* cell line was available for the selection of a recombinant ClGV with an expanded host range. The method undertaken was similar to the complementation assay used by Theim *et al.*, 1996. Overlapping CpGV cosmids or digested CpGV DNA were used to transfect *C. pomonella* cells along with ClGV DNA, Figure 5.1. The idea was to identify a cosmid that rescued ClGV replication in these cells. Subclones of the cosmid would then be used to narrow down the region responsible for rescuing ClGV infection.

The second study in this chapter was initiated to construct occluded recombinant CpGV viruses containing a reporter gene so that the host range and course of infection of CpGV could be investigated. Previously a granulin negative CpGV expressing β-galactosidase had been produced but non-occluded GVs are fragile compared with occluded GVs (D. Winstanley, personal communication).

As well as investigating where in the larvae the CpGV infection starts and how it spreads in its native host *C. pomonella*, it would be interesting to see where the infection

Figure 5.1

CpGV cosmid clones used for transfections and restriction maps of fragments used for transfections.



is blocked in an alternative or non-permissive host. For example, CpGV is very infectious to its native host *C. pomonella*, less infectious to *C. leucotreta* and not at all infectious to *Adoxophyes orana* (D. Winstanley, personal communication).

A transfer vector was constructed containing the *Drosophila* heat shock promoter (*hsp70*) controlling the *Esherichia coli lacZ* gene (*lacZ*). Another transfer vector was constructed containing the late granulin promoter (*gran*) controlling *lacZ*. Both these constructs had CpGV flanking regions for homologous recombination. The regions were chosen so that the *lacZ* gene should recombine and substitute for the ecdysteroid-UDP glucosyl transferase gene (*egt*) which is an auxiliary gene, non-essential for virus replication. Once the recombinant viruses had been selected and purified they would be used to infect the various hosts. The cells and tissues infected could then be visualised by staining with X-gal, a chromogenic indicator for *lacZ* expression. By using these recombinant viruses the course of infection could be studied. The *Drosophila* heat shock promoter will drive expression of the *lacZ* gene as soon as the infection process begins i.e. early gene expression. In contrast, expression from the *gran-lacZ* recombinant would indicate not only that virus was replicating in tissues but that very late viral proteins were being synthesised.

5.2 Results

5.2.1 Studies to identify genes involved in host range determination of CpGV

2×10^6 *C. pomonella* cells (Cp14DWR) were cotransfected with 1 µg ClGV DNA and 5 µg CpGV cosmid DNA (section 2.8.8). Cotransfections were performed using each cosmid separately or all cosmids together. Cells were observed for signs of infection and when confluent, seeded into 96 well plates to allow for further growth and possible infection. The cells from the 96 well plates were dot blotted and probed with a ClGV-specific probe, pCV3HB that was kindly supplied by Dr. J. Jehle (sections 2.5.3 and 2.5.4). This pGEM3Zf (+) plasmid contained a 2.75 kbp ClGV *Eco*RI/*Bam*HI fragment within ClGV *Eco*RI-H, which does not hybridise to CpGV even under low stringency. These experiments were repeated several times but ClGV DNA replication was never detected.

It was thought that this might have been due to the quality of DNA being used for the transfections. Cosmid DNA was always CsCl-density gradient purified (section 2.3.4) but the viral DNA was used freshly extracted from occlusion bodies (section 2.7.4). This was thought to contain a lower proportion of supercoiled DNA. Therefore, the co-transfections were repeated using CsCl-density gradient purified ClGV DNA and only the supercoiled DNA was used in the transfections. However, there were still no signs of ClGV infection and no ClGV DNA replication was detected by dot blotting.

The method was then modified to use ClGV haemolymph rather than DNA; the cells were infected with ClGV haemolymph and then transfected immediately after with CpGV cosmids. As mentioned in the introduction, AcMNPV had been able to enter a wide variety of cell types and a lack of entry into cells did not appear to be the barrier to infection for this virus. Therefore, it was assumed that ClGV budded virus would be able to enter the *C. pomonella* cells. One ml ClGV haemolymph was used to infect 2×10^6 cells and the cells were then transfected with the CpGV cosmids. Again no ClGV replication was detected by dot blotting. A new transfection agent designed for use on insect cells, Insectin-Plus™, was compared to the transfection agent currently

being used which was DOTAP™. CpGV DNA was freshly extracted from OBs and 1 µg was transfected into cells using either DOTAP™ or Insectin-Plus™. The cells were to be alkali lysed and dot blotted to compare the amount of CpGV DNA replication but by 8 d p.i. the cells in the flask transfected with Insectin-Plus™ were virtually completely lysed due to infection and many OBs were observed. In the flask transfected with DOTAP™ only a few areas were beginning to show signs of infection. Therefore, Insectin-Plus™ was clearly a better transfection agent and was used in subsequent experiments.

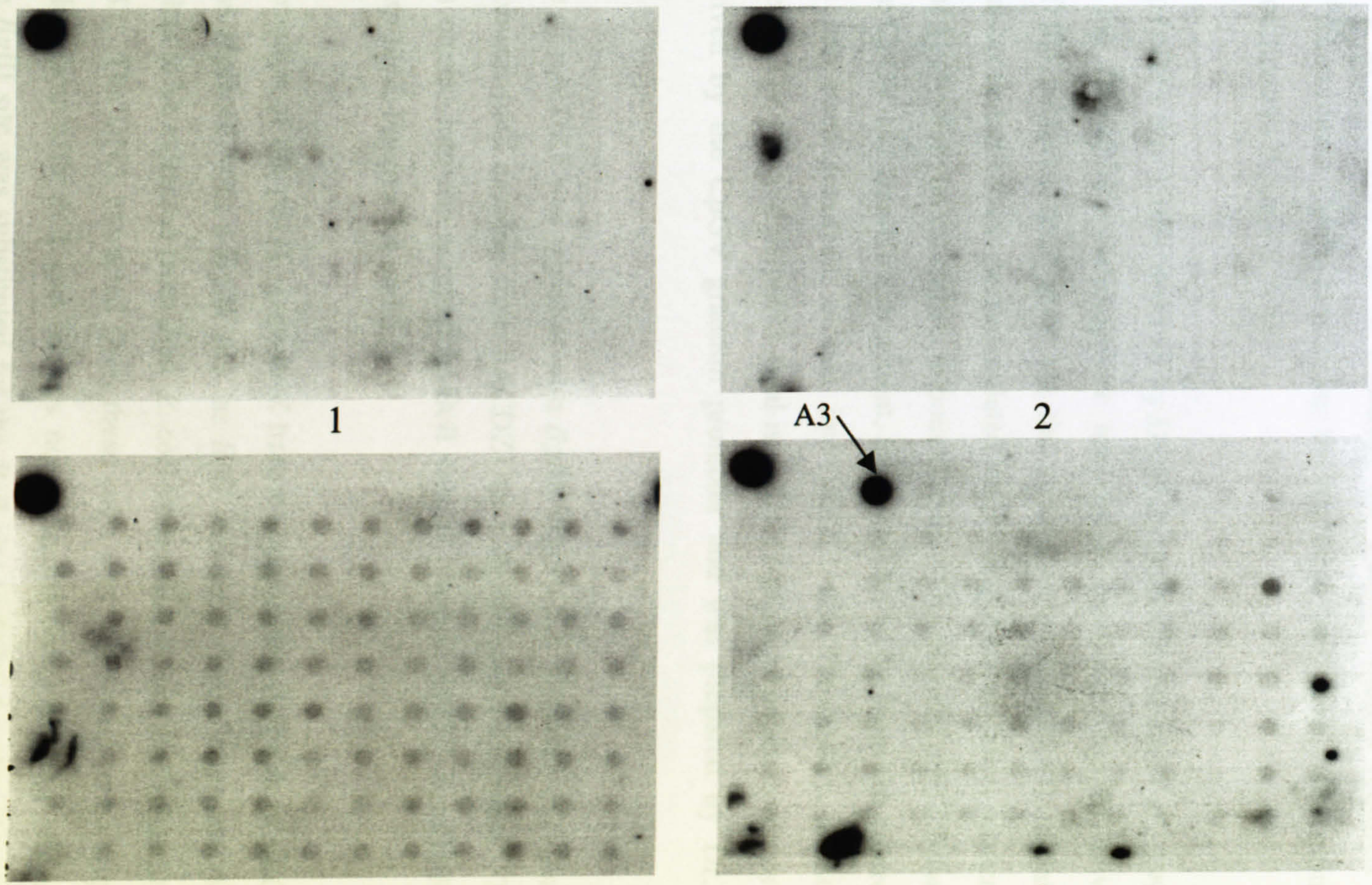
Although the lack of ClGV replication could have been due to poor transfection, it could also have been due to the gene/s responsible for host range expansion spanning across the two cosmids M17 and M69 where there was no overlap between the cosmids, Figure 5.1. Therefore, digested CpGV DNA was used to transfect the cells, along with ClGV DNA (freshly prepared from OBs), supercoiled ClGV DNA (CsCl-density gradient pure) or cells pre-inoculated with ClGV haemolymph prior to transfection with CpGV DNA. A single digest and a double digest of CpGV DNA were prepared using *Sac*I, and *Apa*I plus *Hin*dIII, respectively. The restricted DNA was dephosphorylated to prevent religation of the fragments. Both digests gave fragments of a useful size and the two sets of fragments cut at different points along the genome, Figure 5.1.

The results from these experiments were encouraging. In the cells transfected with ClGV DNA (freshly prepared from OBs) and with CpGV digested with *Sac*I, a ClGV positive well (A3) was detected using the ClGV specific probe, Figure 5.2. This well showed no hybridisation to CpGV and was evidence of ClGV replication in the *C. pomonella* cells. The medium from this well collected at 10 d p.i. was used to inoculate further cells. However, no ClGV DNA replication was detected in these cells. The cells from this well were fed to both *C. leucotreta* and *C. pomonella* fifth instar larvae but no virus-related deaths occurred. The medium from well A3 collected at 6 d p.i. was used to inject both *C. leucotreta* and *C. pomonella* fifth instar larvae but did not result in infection. Finally, the medium from the original transfected culture was used to infect further cells but again no ClGV DNA

Figure 5.2

Dot blots of Cp14DWR cells probed with a ClGV specific probe, pCV3HB. Top left hand corner is 10 ng ClGV DNA. Bottom left corner is 10 ng CpGV DNA. Hybridisation was performed at high stringency (65°C).

1 = mock infected cells
2 = CpGVdigested with *Sac*I, transfected cells
3 = ClGV transfected cells
4 = ClGV DNA + CpGV digested with *Sac*I cotransfected cells



replication was detected. The experiment using ClGV DNA freshly extracted from OBs and CpGV digested with *Sac*I was repeated several times but with no success.

5.2.2 CpGV pathogenesis in various hosts

The initial step of this work was to construct the transfer vectors required for production of recombinant CpGV containing the bacterial *lacZ* gene. The cloning strategies are outlined in Figures 5.3-5.8.

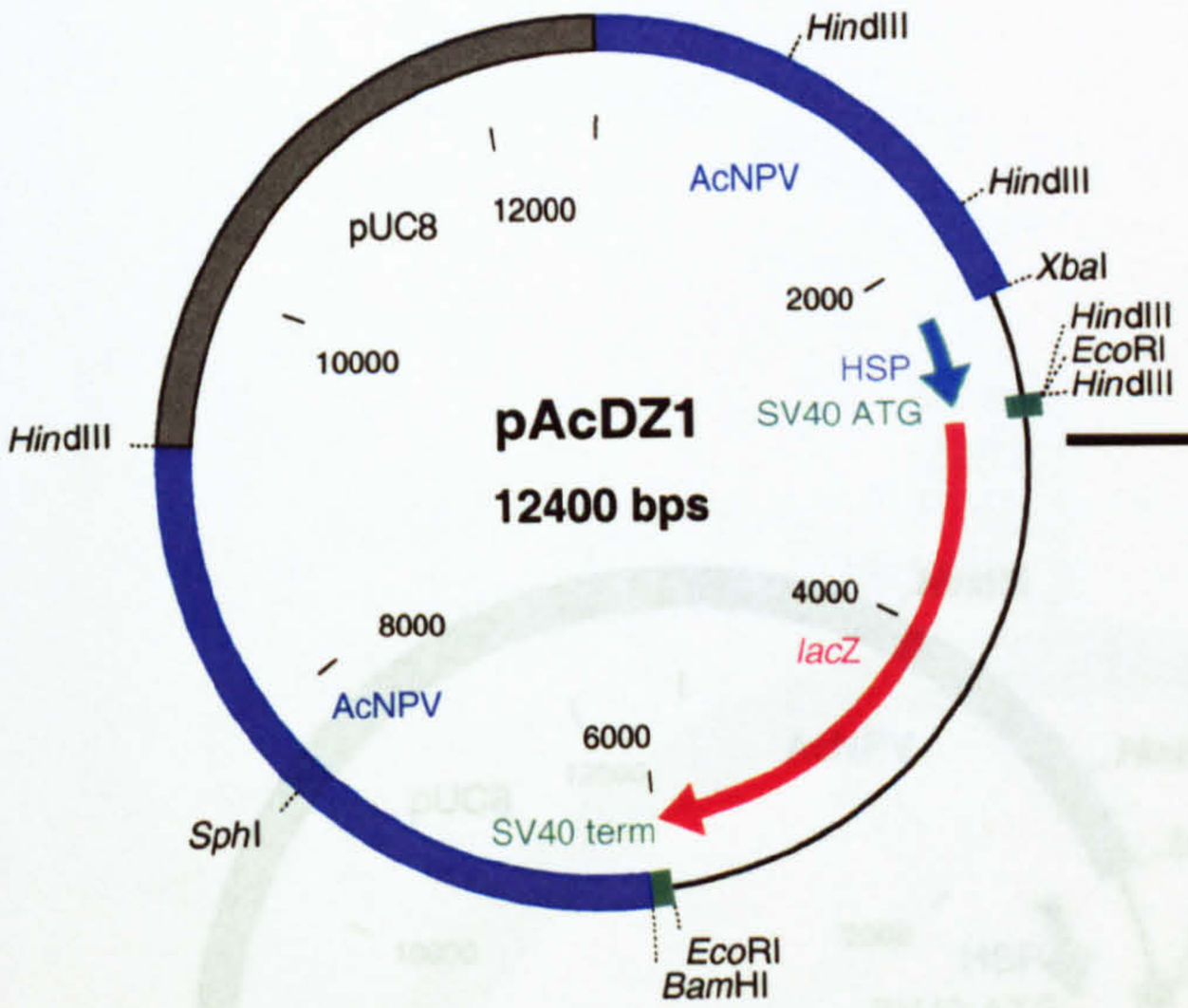
The plasmid pAcDZ1 was kindly donated by Dr. D Zuidema (Zuidema *et al.*, 1990). It contained the *Drosophila melanogaster* heat-shock promoter (*hsp70*) driving expression of the bacterial *lacZ* gene via the simian virus 40 (SV40) initiation sequence. Transcribed mRNA is polyadenylated via SV40 polyadenylation sequence, which contained polyadenylation signals in both strands. The plasmid pEGT⁻*Bam*HI-K contains the CpGV-M1 *Bam*HI-K fragment, from which 753 bp from the centre of the *egt* gene has been removed, cloned into pUC13 (D. Winstanley, personal communication). The plasmid pCpDN1-βgal is a pUC18 plasmid which contains the *lacZ* gene driven by the CpGV granulin promoter and was prepared by Dr. D. Winstanley.

The *Xba*I site of pAcDZ1 was replaced with *Bgl*II, Figure 5.3. allowing excision of the *hsp70* promoter-*lacZ* cassette from pACDZ1 as a *Bgl*II/*Bam*HI fragment. This fragment was cloned into the compatible *Bcl*I site of plasmid pEGT⁻-*Bam*HI-K to form pEGT⁻*hsp-lacZ*, Figures 5.4 and 5.5.

For the construction of the pEGT⁻-*gran-lacZ* transfer vector, the *Bcl*I site of pEGT⁻-*Bam*HI-K was replaced with a *Bgl*II site, as *Bcl*I will only digest non-methylated DNA, which has to be extracted from *dam⁻ E. coli* strains that do not methylate DNA, such as GM33. The DNA extracted from these cells was still found to be slightly resistant to digestion with *Bcl*I and so was not ideal for direct cloning purposes. Therefore the *Bgl*II site was substituted for the *Bcl*I site to form the plasmid pEGT⁻ *Bam*HI-K-*Bgl*II. The plasmid pEGT⁻-*gran-lacZ* was constructed by excising the granulin promoter-*lacZ* cassette from pCpDN1-βgal, exchanging the *Sal*I sites for

Figure 5.3

Cloning strategy of pAcDZ1-*Bgl*II. The *Xba*I site of pACDZ1 was substituted for a *Bgl*II site by blunt ending and ligating *Bgl*II linkers.



Digest with *Xba*I

Blunt end

Ligate *Bgl*II linkers

Religate

Digest with *Bgl*II, *Bam*HI, and *Sph*I

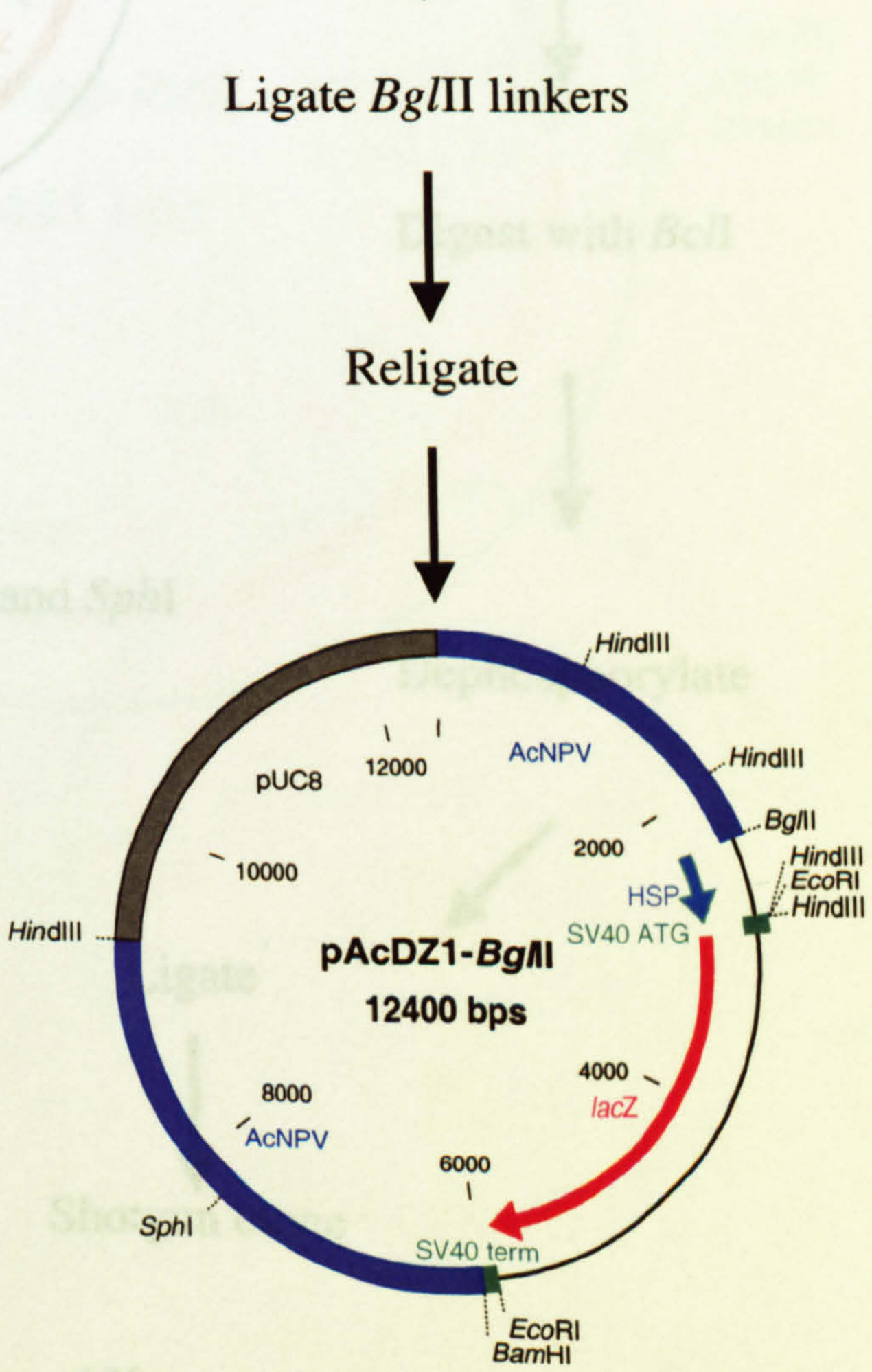


Figure 5.4

Cloning strategy of pEGT⁻-*hsp-lacZ*. The *hsp-LacZ* cassette was released from pACDZ1-*Bgl*II and ligated into the *Bcl*I site of egt-*Bam*H1-K.

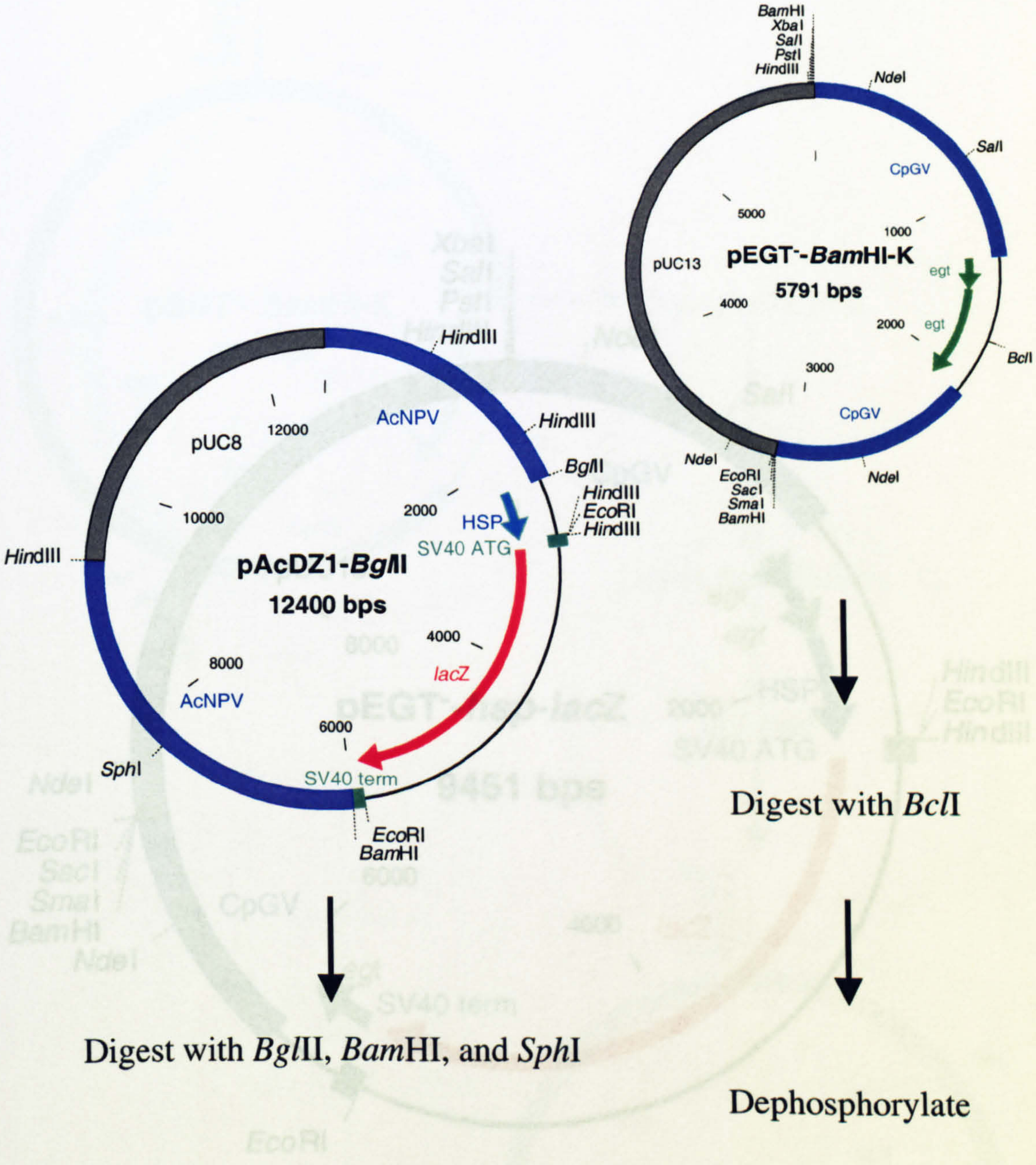


Figure 5.5

Transfer vector pEGT^{-}-*hsp-lacZ*. Constructed via Figure 5.3 and 5.4.

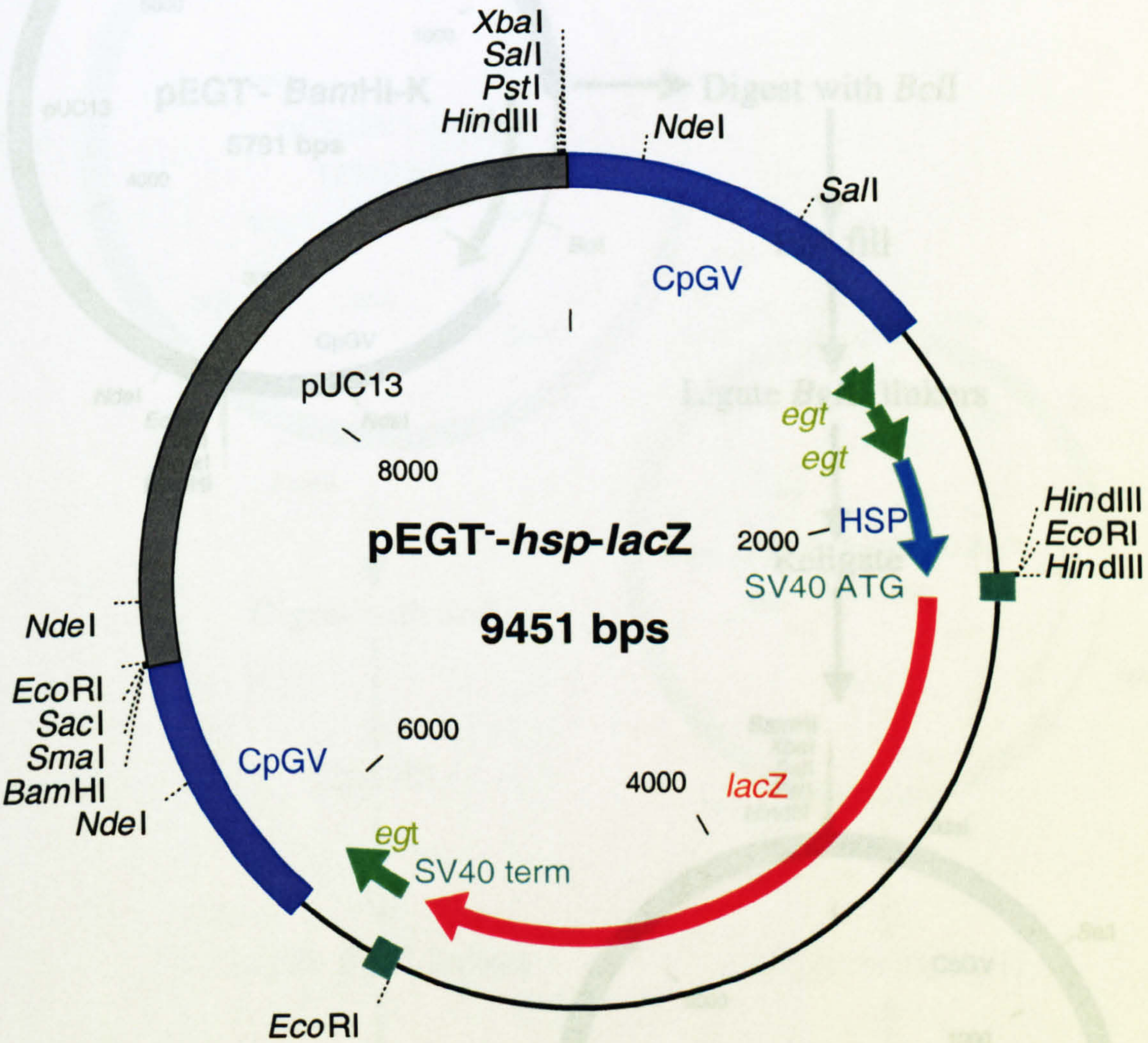


Figure 5.6

Cloning strategy of pEGT⁻-*Bam*HI-K-*Bgl*II. The *Bcl*I site of egt⁻-*Bam*HI-K was substituted for a *Bgl*II site by end filling and ligating *Bgl*II linkers.

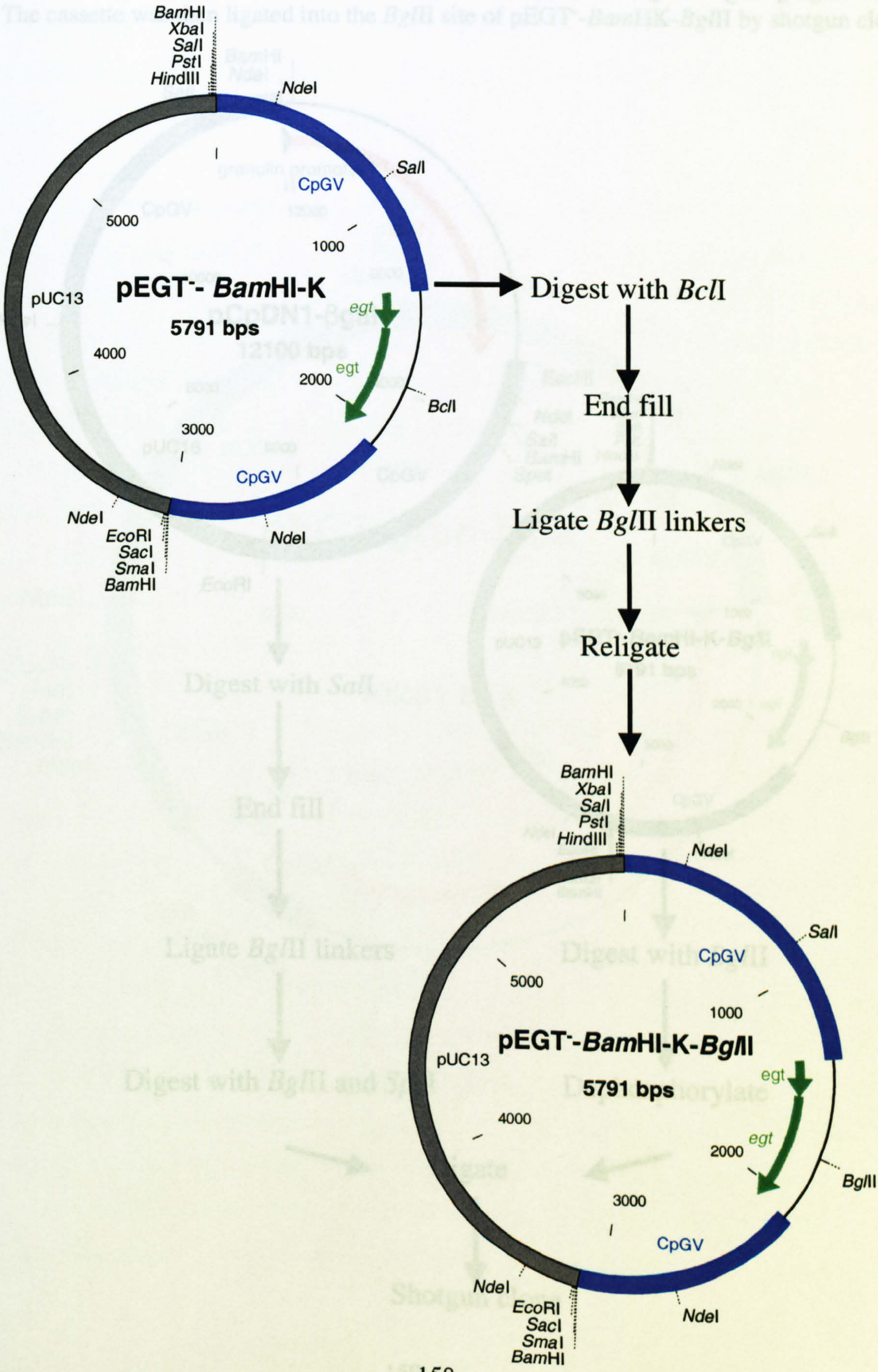


Figure 5.7

Construction of pEGT⁻-*gran-lacZ*. The *gran-lacZ* cassette was released from pCpDN1-βgal. The *Sal*I sites were substituted for *Bgl*II sites by end filling and ligating *Bgl*II linkers. The cassette was then ligated into the *Bgl*II site of pEGT⁻-*Bam*HK-*Bgl*II by shotgun cloning

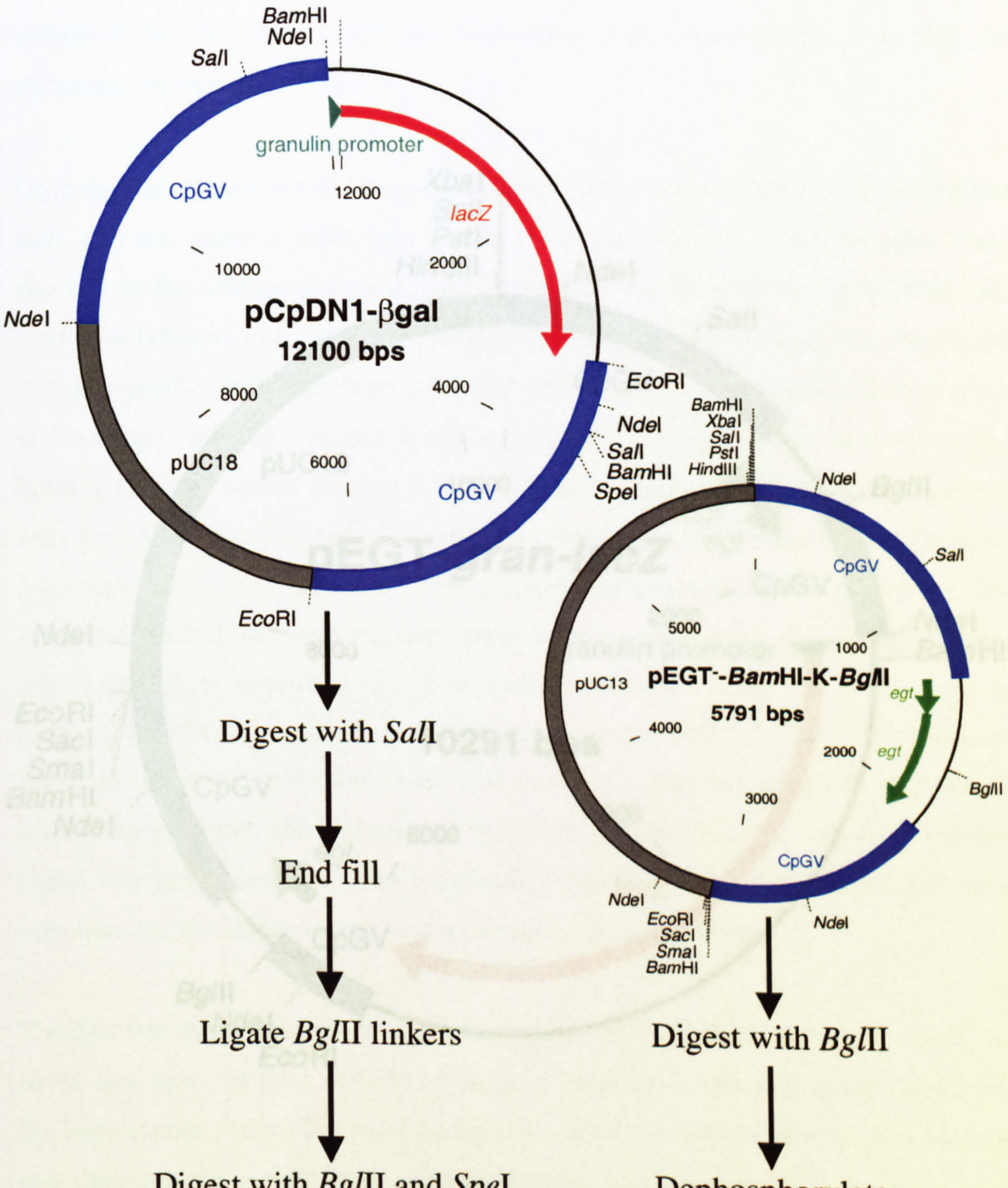
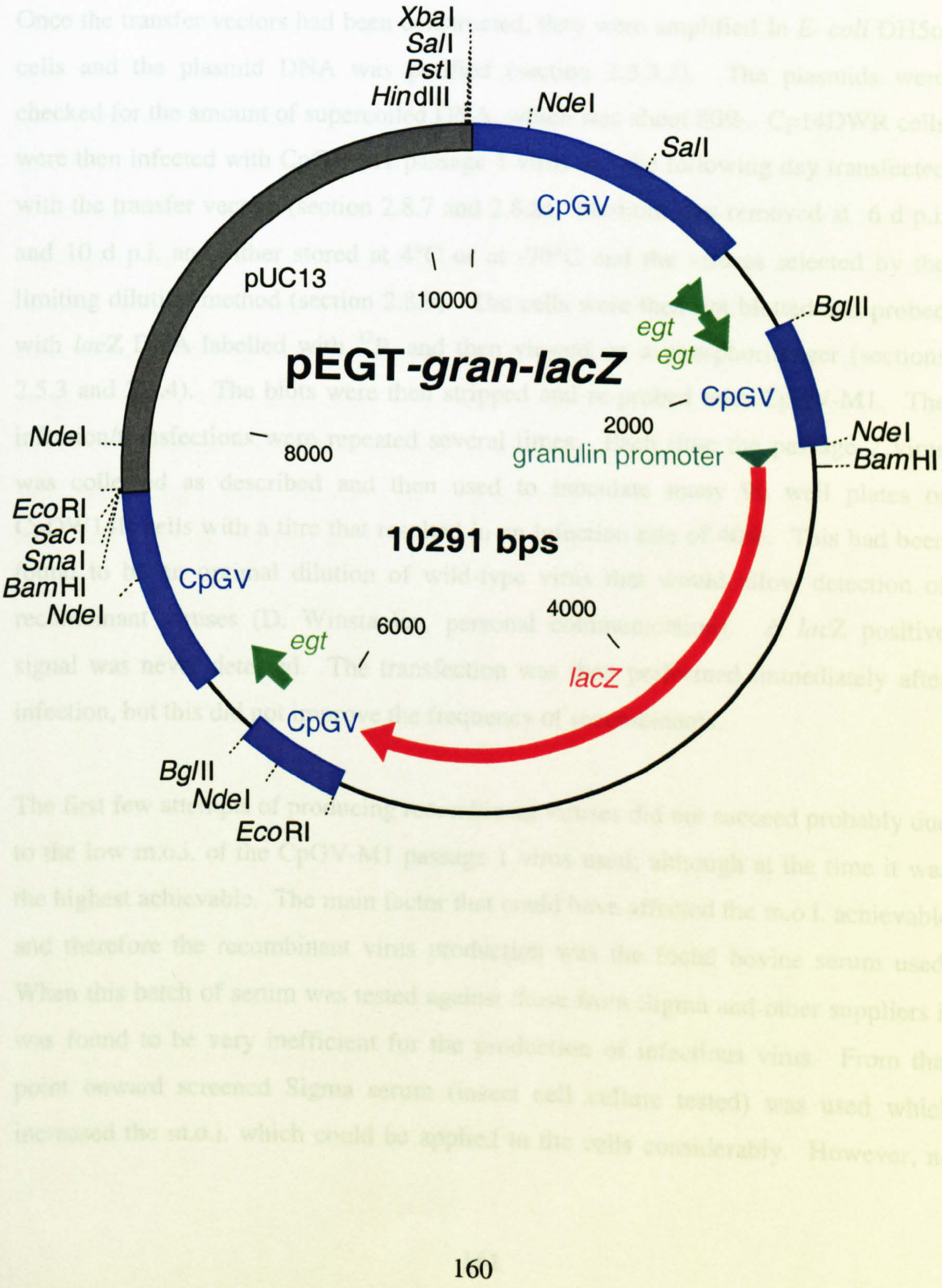


Figure 5.8
Transfer vector pEGT⁻-*gran-lacZ*. Constructed via Figures 5.6 and 5.7.



*Bgl*II sites and cloning into the *Bgl*II site of *egt*⁻-*Bam*HI-K-*Bgl*II, Figure 5.7. The plasmid pEGT ⁻-*gran-lacZ* had small regions of flanking DNA from around the granulin gene, Figure 5.8. However, these should not have been a problem, as any recombination events involving these sites would result in an OB negative virus which would be easily distinguishable from the required recombinants and out-competed by the OB positive recombinants. Also, OB-negative virus can be eliminated by freezing at -20°C.

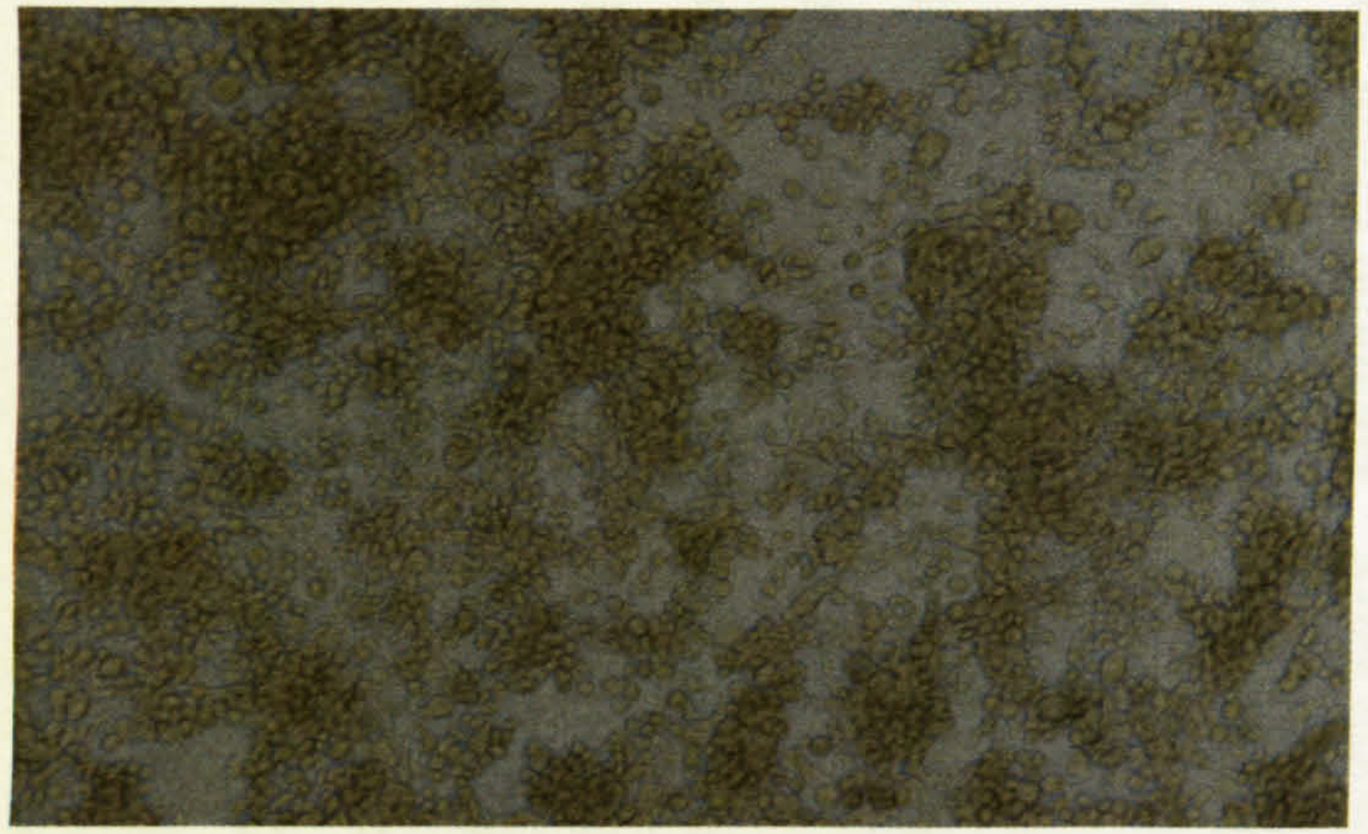
Once the transfer vectors had been constructed, they were amplified In *E. coli* DH5α cells and the plasmid DNA was purified (section 2.3.3.3). The plasmids were checked for the amount of supercoiled DNA, which was about 80%. Cp14DWR cells were then infected with CpGV-M1 passage 1 virus and the following day transfected with the transfer vectors (section 2.8.7 and 2.8.8). Medium was removed at 6 d p.i. and 10 d p.i. and either stored at 4°C or at -70°C and the viruses selected by the limiting dilution method (section 2.8.9). The cells were then dot blotted and probed with *lac*Z DNA labelled with ^{32}P, and then viewed on a phosphorimager (sections 2.5.3 and 2.5.4). The blots were then stripped and re-probed with CpGV-M1. The infection/transfections were repeated several times. Each time the passage 2 virus was collected as described and then used to inoculate many 96 well plates of CpDW14R cells with a titre that resulted in an infection rate of 40%. This had been found to be an optimal dilution of wild-type virus that would allow detection of recombinant viruses (D. Winstanley, personal communication). A *lac*Z positive signal was never detected. The transfection was then performed immediately after infection, but this did not improve the frequency of recombinants.

The first few attempts of producing recombinant viruses did not succeed probably due to the low m.o.i. of the CpGV-M1 passage 1 virus used; although at the time it was the highest achievable. The main factor that could have affected the m.o.i. achievable and therefore the recombinant virus production was the foetal bovine serum used. When this batch of serum was tested against those from Sigma and other suppliers it was found to be very inefficient for the production of infectious virus. From that point onward screened Sigma serum (insect cell culture tested) was used which increased the m.o.i. which could be applied to the cells considerably. However, no

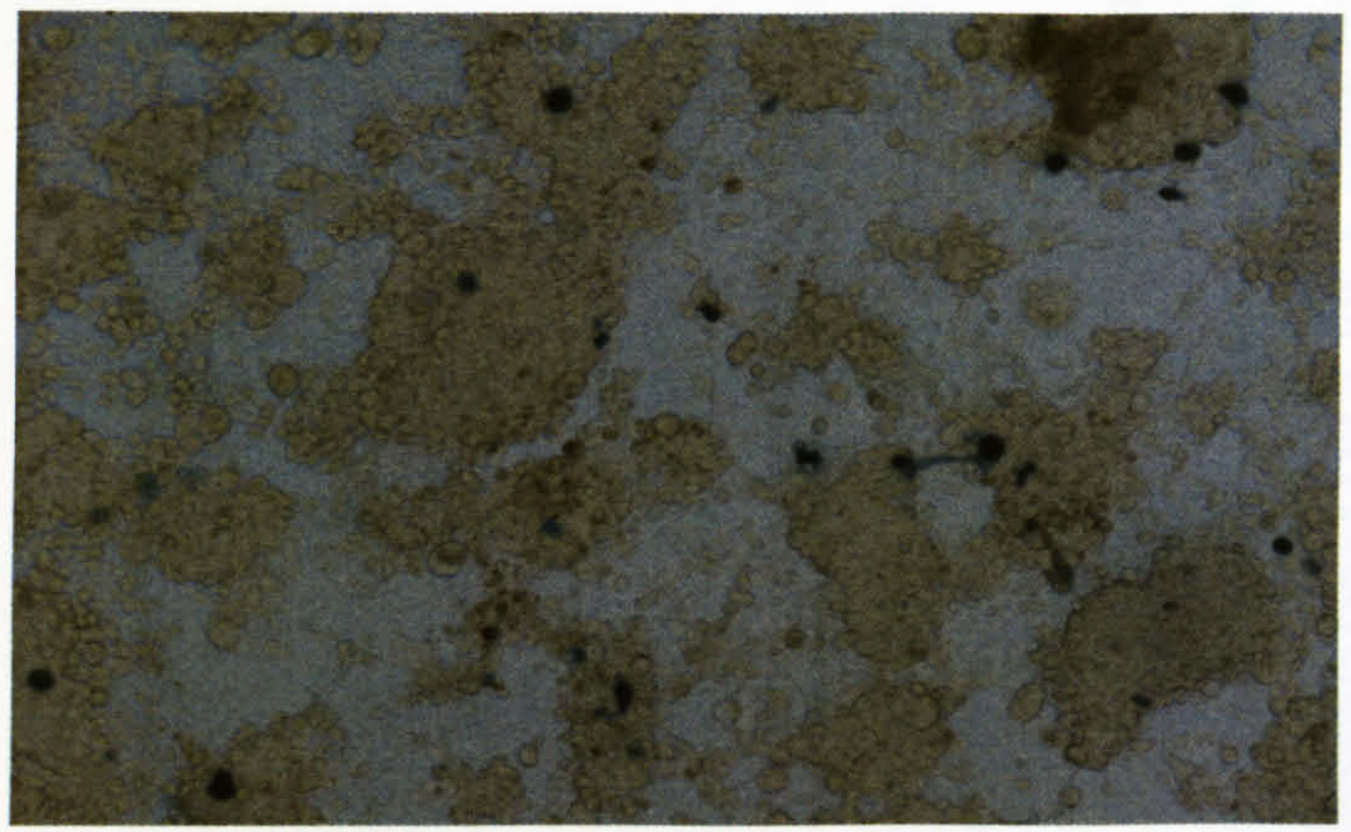
recombinants were recovered. A poor transfection efficiency was another possible reason for the lack of recombinants. Therefore, this was investigated. The transfection efficiency of Insectin-Plus™ was compared to a number of different transfection agents by transfection of the reporter plasmid pEGT-*hsp70-lacZ* then fixing and staining the cells with X-gal. The efficiency of transfection was indicated by the degree of β-galactosidase expressed in the transfected cells. All of the transfection reagents appeared very inefficient with only one or two cells per flask turning blue i.e. expressing β-galactosidase. In most flasks no blue cells were observed, suggesting a poor transfection efficiency. However, there was efficient uptake of the plasmid as judged from a relatively strong signal obtained, using the *lac*Z probe observed on dot blots from transfected cells. In addition, adding X-gal to medium from cells transfected with a *lac*Z plasmid resulted in only a very slight blue coloration after incubation at 37°C overnight indicating poor β-galactosidase expression and possibly a low transfection efficiency. In contrast, medium from cells infected with CpGV before transfection with a *lac*Z plasmid gave a strong blue coloration, indicating high β-galactosidase expression. This prompted additional experiments in which cells were fixed and stained with X-gal after infection with CpGV-M1 passage 1 virus and transfection with the reporter plasmid. The number of cells expressing β-galactosidase were recorded and compared to the number of blue cells in cells which had only been transfected, Table 5.1; Figure 5.9.

Figure 5.9
CpDW14R cells fixed and stained with X-gal 48 hours after infection with CpGV-M1 passage 1 virus for four hours and/or transfection with 5 μg reporter plasmid immediately after for four hours.

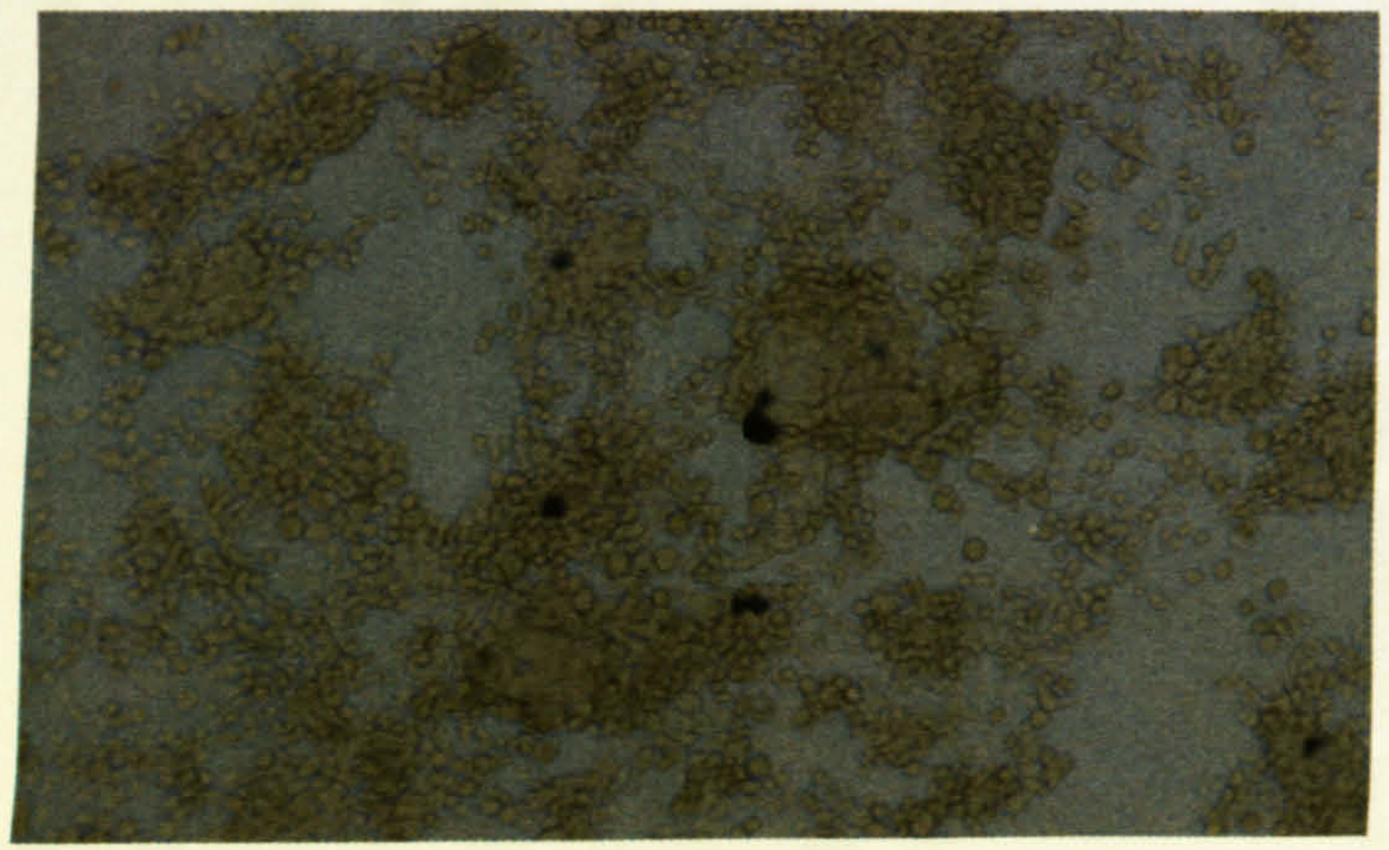
1= Transfected with pEGT -*gran-lacZ*
2= Transfected with pEGT -*gran-lacZ* and infected with CpGV
3= Transfected with pEGT -*hsp-lacZ*
4= Transfected with pEGT -*hsp-lacZ* and infected with CpGV



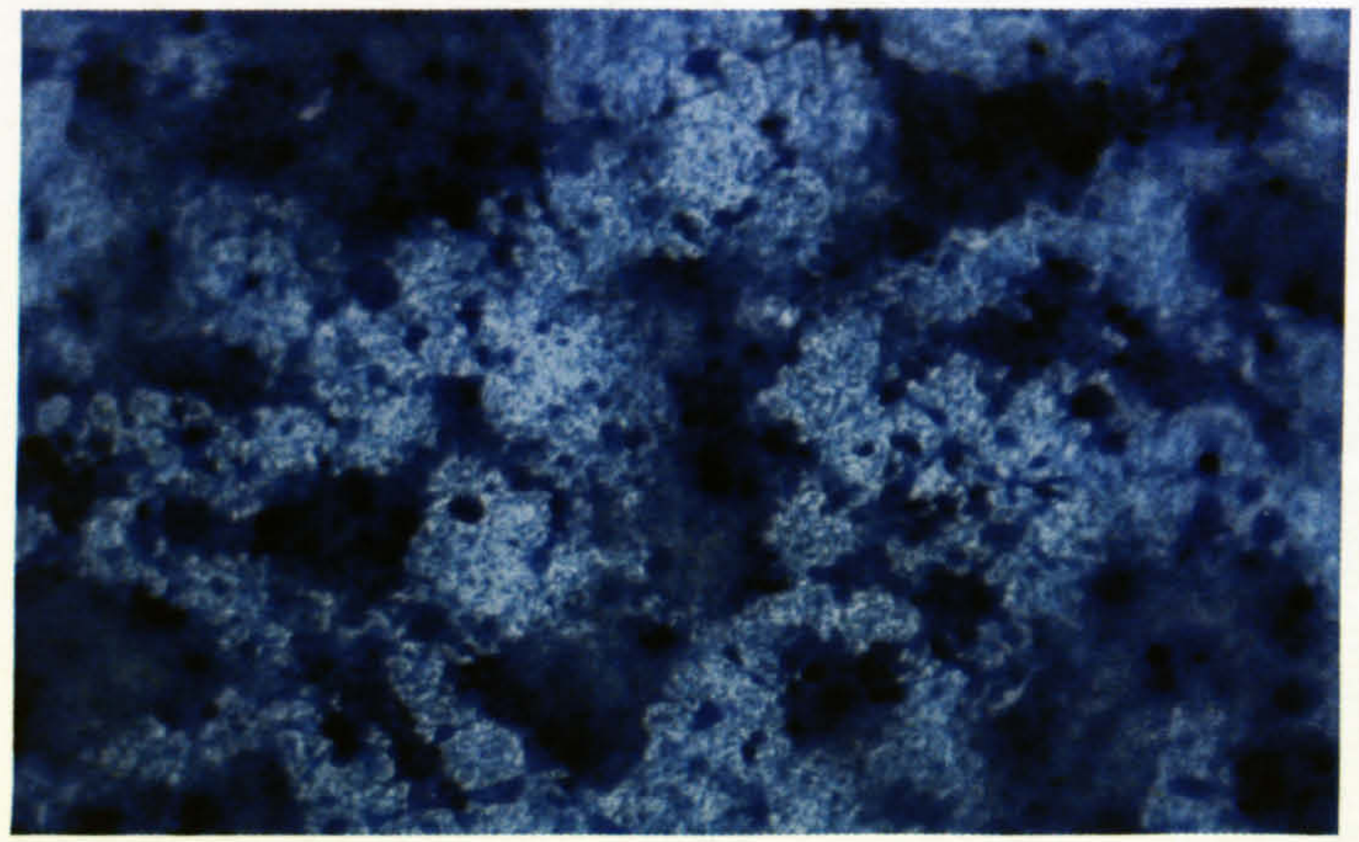
1



2



3



4

Table 5.1

The percentage of Cp14DWR cells that expressed β-galactosidase after fixing and staining with X-gal, after different infection/transfections. Infected/transfected cells were infected with 1 ml CpGV-M1 passage 1 virus for 4 hours then immediately transfected with 5 μg reporter plasmid for 4 hours. The cells were fixed and stained 48 hours later.

Cells	% blue cells (transfection efficiency)
Mock infected	0
pEGT¯-*hsp70-lacZ*	0.0006
pEGT¯-*hsp70-lacZ* + CpGV	67
pEGT¯-*gran-lacZ*	0
pEGT¯-*gran-lacZ* + CpGV	4.3
CpGV	0

These data confirmed that the cells were being transfected very efficiently but that pre-infection with CpGV was required for efficient expression of β-galactosidase, even from the *hsp70* promoter. After the infection efficiency was improved using a suitable batch of FBS and the transfection efficiency was confirmed, the recombination experiment was repeated.

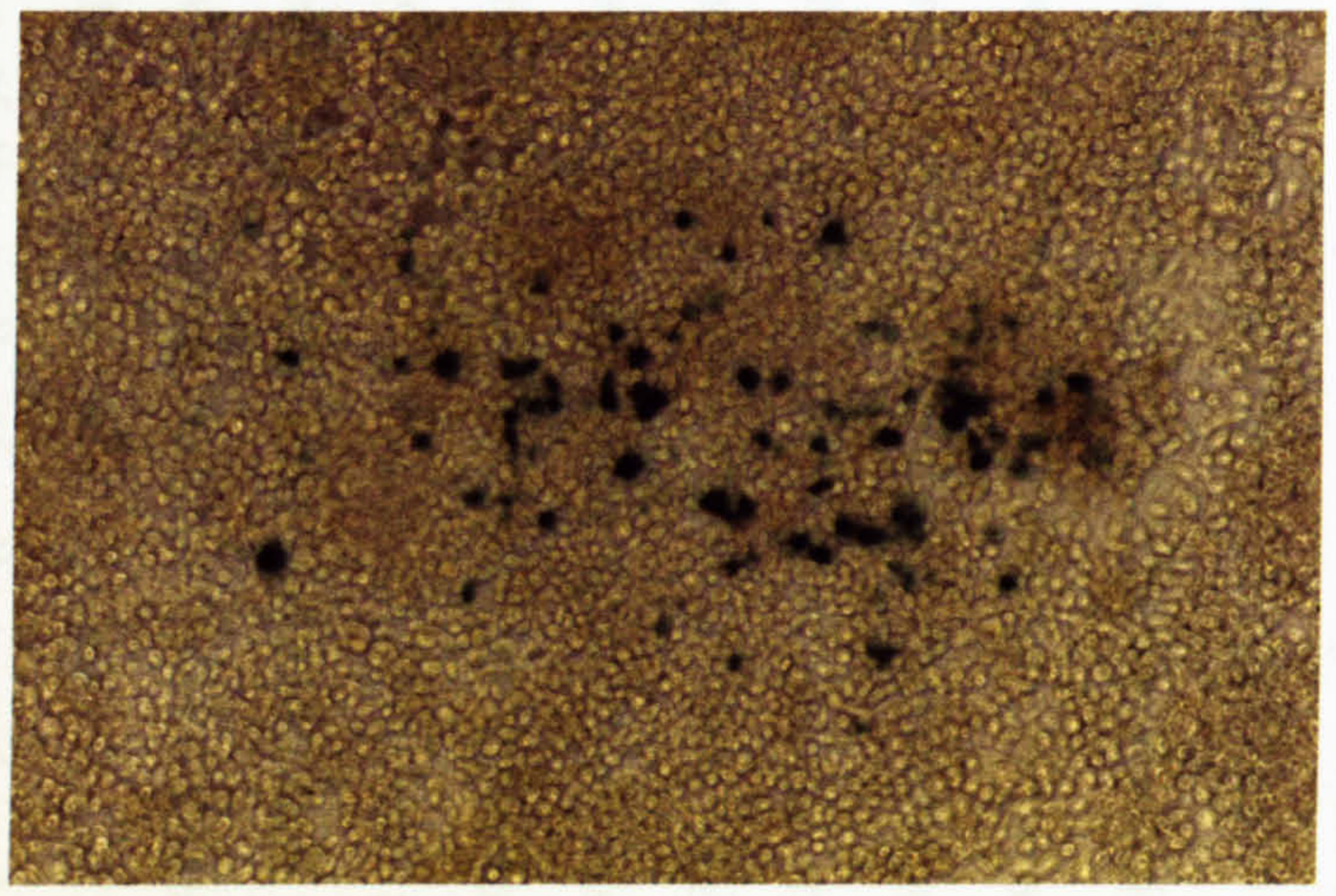
The method of detection of recombinant virus was also reviewed. A few different methods were investigated, as dot blotting may not have been sensitive enough to detect the small amount of recombinant virus that may have arisen. Cells were infected with CpGV-M1 P1 virus then transfected with either of the transfer vectors. Medium was removed 7 d p.i. and one 96-well plate was infected with a dilution of the P2 virus from 1/50 to 1/1600. This was to determine the dilution of virus required to infect 40% cells. It was found to be a 1/200 dilution for P2 virus. Therefore, five 96 well plates of cells were infected with 1/200 dilution of the P2 virus from either pEGT¯-*hsp70-lacZ* or *gran-lacZ* infections/transfections. The cell density in these wells was a bit too high and as a result not much infection could be seen by 10 d p.i. Three plates were analysed by dot blot and probed with the *lacZ* probe; one plate was

stained with X-gal and one was to be used to amplify *lac*Z DNA by PCR. The dot blots all came up negative using the *lacZ* probe. The stained pEGT¯*-hsp70-lacZ* 96 well plate was very promising as one well contained a patch of blue cells which must have been recombinant virus spreading among the cells, Figure 5.10. One other well contained one blue cell. The pEGT¯*-gran-lacZ* plate did not have any blue cells but this was not surprising as the infection was obviously at an early stage because of the slow rate of infection in the cells. More time would have allowed development of these results further and enabled PCR of the cell DNA to be carried out which would also be a very sensitive method of detection of recombinant virus.

Figure 5.10

Cp14DWR cells infected with P2 medium from 1, *hsp70-lacZ* transfected cells and 2, *hsp-lacZ* transfected and CpGV-M1 infected cells. The patch of blue cells indicates the presence of the *hsp-lacZ* recombinant virus.

1



2

5.3 Discussion

5.3.1 Studies to identify genes of CpGV involved in host range determination

The attempts to rescue ClGV replication in *C. pomonella* cells met with limited success. The transfection agent originally used (DOTAP™ Roche Diagnostics) was an updated version of a transfection agent that had previously been optimised to give good transfection efficiencies (DOTMA™ Roche Diagnostics). Clearly the new product was not as good for this cell line. Subsequent use of Insectin-Plus™ considerably increased the transfection efficiencies and the chances of recombination. Viral DNA that had been freshly extracted from OBs may have contained a low proportion of supercoiled DNA. Supercoiled viral DNA has been shown to be most infectious and nicked circular and linear DNA less infectious for cells. Therefore, supercoiled DNA was purified on a CsCl-density gradient and used in some of the experiments. Haemolymph from *C. leucotreta* infected with ClGV was also used as a possibly more efficient way of ensuring entry of ClGV into the cells. However, it was transfection with the ClGV DNA, freshly extracted from OBs, which resulted in ClGV replication in one of the wells of cells transfected also with CpGV DNA digested with *Sac*I.

A recombinant ClGV could not be recovered in *C. pomonella* cells using the CpGV cosmids. This may have been because the cosmid did not contain the gene or factor responsible. The region of the CpGV genome that is not overlapped by two cosmids is covered by the cosmid M61. Therefore this cosmid should also be used in future studies. However, the replication detected in one well does confirm the ability of the *Sac*I fragment/s of the CpGV genome to complement the ClGV genome, allowing it to replicate in *C. pomonella* cells. Whether or not a recombinant virus was formed is not evident. The ClGV positive well did not hybridise to a CpGV probe and if a fragment of CpGV over a few hundred base pairs had inserted into the genome it would have been expected to hybridise. Also the progeny virus was non-infectious *per os*, by intrahaemocylic injection to *C. pomonella* and *C. leucotreta* larvae and by infection to further *C. pomonella* cells. If a productive infection of ClGV had occurred it would have been infectious to *C. leucotreta* larvae. This suggested an abortive infection, involving DNA replication but not production of infectious

progeny virions. OBs were not observed within the well, nor were any cytopathic effects. However, if ClGV could replicate its DNA in the presence of CpGV DNA then ClGV DNA replication may have been expected in more wells than just the one where it was detected. Therefore it may be possible that recombination did occur with a very small amount of CpGV DNA that allowed DNA replication but was not enough to be detected by hybridisation.

In theory, multiple recombination events probably occurred during these experiments but only this one allowed replication of ClGV DNA in the *C. pomonella* cells. High m.o.i.s of GV in *C. pomonella* cells cannot readily be achieved, so this cell system is not as efficient as NPV recombination systems. Therefore, the chances of selecting a recombinant in this system were quite small. Another factor that could have affected the outcome of the experiment was the foetal bovine serum used, which was mentioned in 5.2.2. The experiment will be repeated using cells adapted to the new foetal bovine serum. Now that the cell culture system is enhanced, the probability of recovering recombinant viruses with an expanded host range should be greatly improved.

5.3.2 CpGV pathogenesis in various hosts

The sensitivity of detection of recombinants appeared to be the limiting factor in this study. Although dot blotting has previously been found to be adequate in detecting recombinants, in some cases more sensitive techniques may be required. The lack of hybridisation gave the impression that there was no recombination. Therefore, the transfection agent was investigated. This led to the conclusion that nothing was wrong except that recombination was occurring at a very low efficiency and a more sensitive detection procedure was required. As the recombinants expressed β-galactosidase, fixing and staining with X-gal seemed like a suitable method. It was noticed during this study that the transfer vectors would only express β-galactosidase if the cells had been infected with CpGV. The promoter region of the *lacZ* gene may require some gene products from the CpGV genome to transactivate it. This may have been expected for the late granulin promoter, as it is a promoter from the CpGV genome. However, for the *hsp70* promoter a requirement for CpGV proteins was

unexpected. Possible explanations are that CpGV DNA contains a transactivating factor for the *hsp70* promoter or that the host cell DNA encodes a transactivating factor that is activated in some way by CpGV replication. It may be a host cell response to viral infection that increases the expression of an infected cell specific protein, which transactivates the *hsp70* promoter. The *hsp70* promoter is up-regulated by an increase in temperature as a cell defence mechanism. Therefore, the infection of a cell may initiate a similar cell defence mechanism and up-regulate the *hsp70* promoter.

This experiment proved that the transfection efficiency of *C. pomonella* cells was very good. The infection efficiency was dramatically affected by the batch of foetal bovine serum used and a better batch was selected. By fixing and staining cells that were infected with medium from the transfected/infected culture, recombinant viruses could be detected. Any cells expressing the reporter gene would have to contain a recombinant virus, as there would not be any *lacZ*-plasmid in the medium due to medium changes on the transfected/infected cells. Blue cells were detected in two wells out of 96. A small patch of infection was observed in one, which indicated the spread of a recombinant virus (Figure 5.10). This amount of recombinant virus would probably not have been detected by dot blotting. The medium from this well has been stored at –70°C and will be used in an attempt to clone the virus. A similar method could be used to obtain a *gran-lacZ* recombinant virus, although the cells should not be stained until OBs are readily observed as β-galactosidase will not be expressed until late in infection. PCR would be another way of detecting small amounts of recombinants. Primers have been designed that amplify a piece of DNA within the *lacZ* gene. The advantage of using PCR is that the amount of wild-type virus can be estimated by PCR using CpGV primers that amplify a region within the *egt* gene that is interrupted by the insertion of the *lacZ* cassette. This would distinguish the wild-type from the recombinant. Both of these methods should be used in the future cloning of these viruses. The cloned viruses will then be used to study the course of infection through the various hosts.

A time course study could be performed to see if the infection is arrested at a particular site in the semi-permissive host and to tell when and where the infection is

blocked in semi-permissive and non-permissive hosts. The vector could also be used to carry further inserted genes required to increase pathogenicity, for improving biopesticides. The inclusion of a *lacZ* gene would make the recombinant virus easier to select and extensive host range studies could be performed to check that the insertion had not increased the host range to non-target pests. Another reporter gene that could be inserted into the *egt* gene would be the green fluorescent protein (GFP) which was isolated from the bioluminescent jellyfish *Aequorea victoria*. This protein, when expressed and exposed to blue or UV light, emits an intense green fluorescence. The advantages of using this protein are that it does not require cofactors or substrates and can be visualised in living tissues (Chalfie *et al.*, 1994; Wilson *et al.*, 1997). Therefore larvae would not need to be fixed and stained to observe infection. Also, selection of recombinant viruses would be easier as the fluorescent cells could be fed directly to larvae to bulk the recombinant virus up *in vivo*.

CHAPTER 6

The effect of a 2.45 kbp region in the CpGV-M1 genotype on speed of kill

6.1 Introduction

Isolates of baculoviruses from the field are often a mixture of different genotypes and it is important to characterise these viruses since different genotypes may be more efficacious than others. Individual genotypes can be cloned either *in vivo* or *in vitro*. Once cloned genotypes are obtained, comparisons can be made and differences that may be advantageous to their use as biocontrol agents may be observed. These include the ability to replicate efficiently in cell culture, the amount of virus produced in larvae, their host range, lethal dose and speed of kill.

To date, several genotypes of CpGV have been found to infect the lepidopteran pest *Cydia pomonella.* CpGV-M1 is a genotype cloned *in vivo* from the Mexican isolate of CpGV (CpGV-M). It is the genotype that has been studied most and the sequencing of its genome is near completion. CpGV-R1 is a genotype cloned from the Russian isolate of CpGV. Preliminary studies using bioassays to compare CpGV-R1 and CpGV-M showed that CpGV-M killed neonate larvae approximately 20% faster than CpGV-R1, although details of the experiment were not given (Chowdhury, 1992). Previous studies had identified a 2.4 kbp insert in CpGV-M1, as well as unrelated restriction site differences, compared with CpGV-R1 (Crook, 1991). As the insert was the major difference between the two genotypes it seemed possible that this region may be responsible for the increased speed of kill of CpGV-M1. If so, the region could eventually be inserted into other baculovirus genomes to attempt to alter their speed of kill.

The aim of the work described in this chapter was to produce a recombinant CpGV-R1 virus, which contained the extra region from CpGV-M1 DNA. Bioassays performed on neonate larvae (the targets for any effective biocontrol) using the recombinant virus alongside CpGV-M1 and CpGV-R1 would determine if the extra region of DNA was responsible for the increased speed of kill of the CpGV-M1 genotype.

6.2 Results

6.2.1 CpGV-R1/CpGV-M1 *Sal*I-F recombination

CpGV-R1 has 2451 bp deleted from the *Sal*I-F fragment compared to CpGV-M1. It also contains small insertions of up to 11 bp and deletions of up to 62 bp in the kilobase of DNA to the left of the deletion, Appendix 10. The 780 bp of DNA to the right of the deletion has only a few base pair substitutions compared with CpGV-M1, Appendix 10.

Primers were designed either side of the deleted region to amplify 3646 bp in CpGV-M1 and 1195 bp in CpGV R1, Figure 6.1. These were named REPT1 and REPT2 and their nucleotide sequences are shown below.

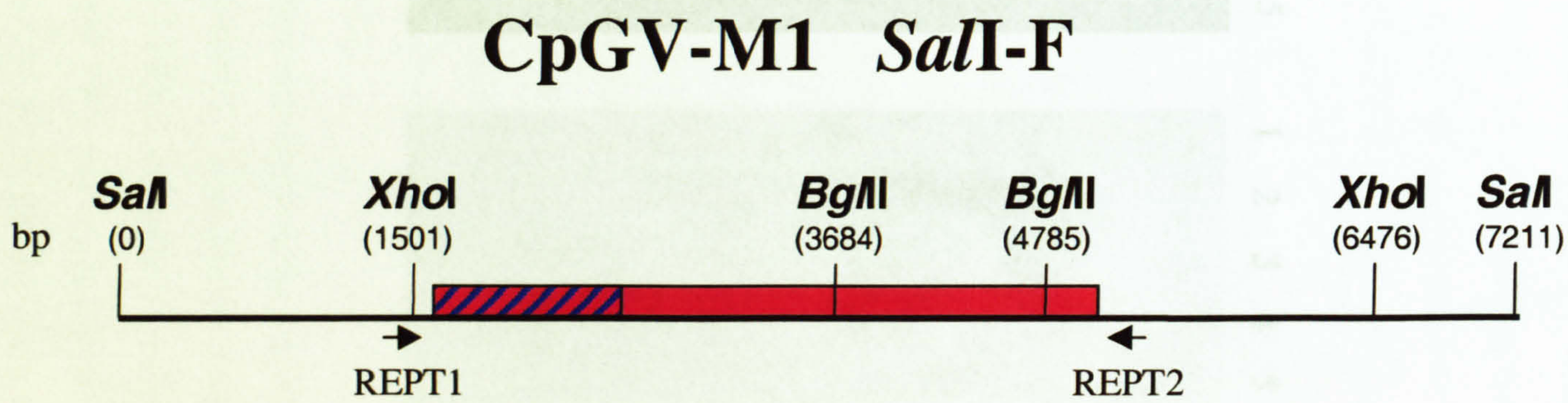
Upper primer	REPT1	5' GAGAGTGTGCGCGGTTGCTGAGATA 3'
Lower primer	REPT2	5' CGAAGTCACAGCAGGTCCATAGA 3'

CpGV-M1 *Sal*I-F had been previously cloned into the plasmid pBSK+. Purified DNA was digested with *Sal*I to check the profile and undigested plasmid was run on an agarose gel to check the amount of supercoiled DNA (sections 2.2.1 and 2.2.2). Approximately 75% of the DNA was supercoiled and the restriction endonuclease profiles were correct.

The OBs from two fifth instar *C. pomonella* larvae which had died from CpGV-R1 infection were individually purified and DNA extracted (sections 2.7.1.2 and 2.7.4). The restriction endonuclease profiles were correct for CpGV-R1 with no submolar bands and there was no hybridisation to the *Bgl*II-G fragment present in CpGV-M1 *Sal*I-F on Southern blotting (section 2.5.1), Figure 6.2.

All larvae used in this study were *C. pomonella* and from here on will be referred to only as larvae. The OBs from one of the larvae infected with CpGV-R1 were used for the production of infectious haemolymph (section 2.8.6).

Figure 6.1
Restriction map of CpGV-M1 *Sal*I-F showing the region of CpGV-M1 absent from CpGV-R1 and the upstream area containing other sequence differences between the two genotypes. REPT1 and REPT2 primers used for the amplification of CpGV-M1 and CpGV-R1 are shown below the map.



= Region absent from CpGV-R1

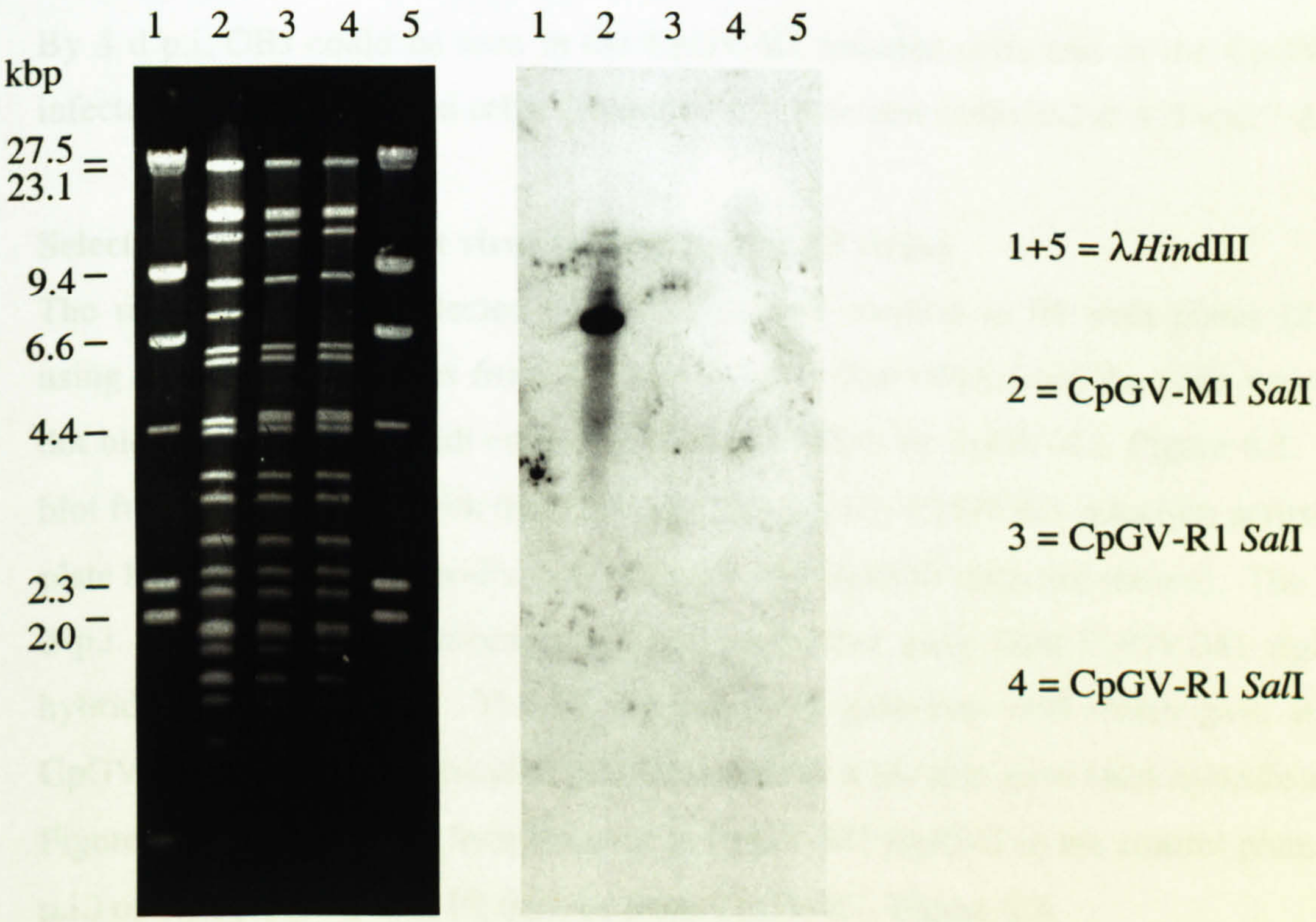
= Region containing differences between CpGV-R1 and CpGV-M1

→ = Primers

Figure 6.2

Left panel: 0.7 % agarose gel of CpGV-M1 positive control DNA and DNA from two CpGV-R1 infected larvae, digested with *Sal*I.

Right panel: Autoradiograph of gel probed with CpGV-M1 *Bgl*II-G at high stringency (65°C).



Production of passage 1 (P1) virus inoculum

Passage 1 virus produced in CpDW14R cells from CpGV-R1 haemolymph was used in this experiment (section 2.8.6).

Infection and transfection of *C. pomonella* cells (production of P2 virus)

CpDW14R cells were plated out at 2 x 10^6 cells per small flask. The cells were infected with 1 ml CpGV-R1 P1 virus and then transfected using Insectin™ with 5 µg CpGV-M1 *Sal*I-F DNA (sections 2.8.7 and 2.8.8). Controls used were mock infected cells, cells infected with CpGV-R1 P1 and cells transfected with *Sal*I-F DNA.

By 4 d p.i. OBs could be seen in the CpGV-R1 infected cells and in the CpGV-R1 infected/ *Sal*I-F transfected cells. Therefore P2 virus was collected at 4, 5 and 7 d p.i.

Selection of recombinant virus (production of P3 virus)

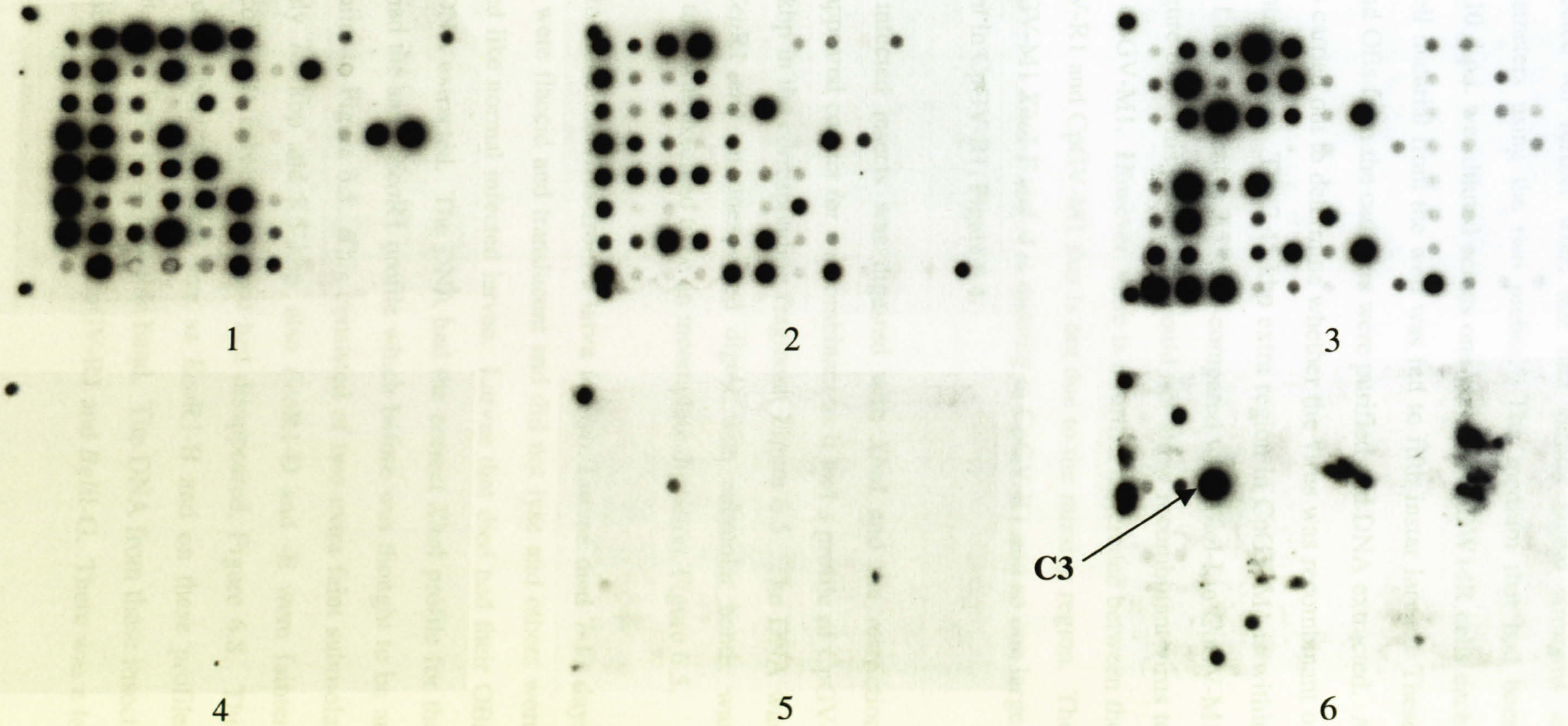
The recombinant was selected using the dilution method in 96 well plates (2.8.9) using dilutions of P2 virus from 4, 5 and 7 d p.i. The DNA from the cells was then dot blotted and probed with either CpGV-M1 *Bgl*II-G or CpGV-R1, Figure 6.3. The blot from cells infected with the P2 4 d p.i. stock gave CpGV-R1 infection across the plate but there was no hybridisation to CpGV-M1 *Bgl*II-G (data not shown). The P2 5 d p.i. stock gave more infection and two wells that gave faint CpGV-M1 *Bgl*II-G hybridisation, Figure 6.3. The P2 7 d p.i. stock gave one well which gave strong CpGV-M1 *Bgl*II-G hybridisation (C3) and several wells that gave faint hybridisation, Figure 6.3. There was no hybridisation to CpGV-M1 *Bgl*II-G in the control plate (7 d p.i.) of cells infected with P2 derived from CpGV-R1, Figure 6.3.

Second selection of recombinant virus (production of P4 virus)

Medium harvested from well C3 was diluted across a plate of CpDW14R cells. The cells were dot blotted at 10 d p.i. and probed with the CpGV-R1 and CpGV-M1 *Bgl*II-G probes. The blots gave very strong signals when probed with CpGV-M1 *Bgl*II-G and similar strength signals when probed with CpGV-R1 (data not shown). This suggested that the virus present was mostly the recombinant.

Figure 6.3
Dot blots of cells infected with P2 virus from CpGV-R1 infected cells and CpGV-R1 infected/*Sal*I-F transfected cells. The top row (1-3) were probed with CpGV-R1 DNA. The bottom row (4-6) were probed with CpGV-M1 *Bgl*II-G. All medium was diluted from 1/20-1/640 across each plate from left to right. Top left corner of each blot is 5 ng *Sal*I-F DNA. Bottom left corner is 5 ng CpGV-R1 DNA.

1+4 = CpGV-R1 7 d p.i. P2 medium
2+5 = CpGV-R1 + *Sal*I-F 5 d p.i. P2 medium
3+6 = CpGV-R1 + *Sal*I-F 7 d p.i. P2 medium



Third selection of recombinant virus (production of P5 virus)

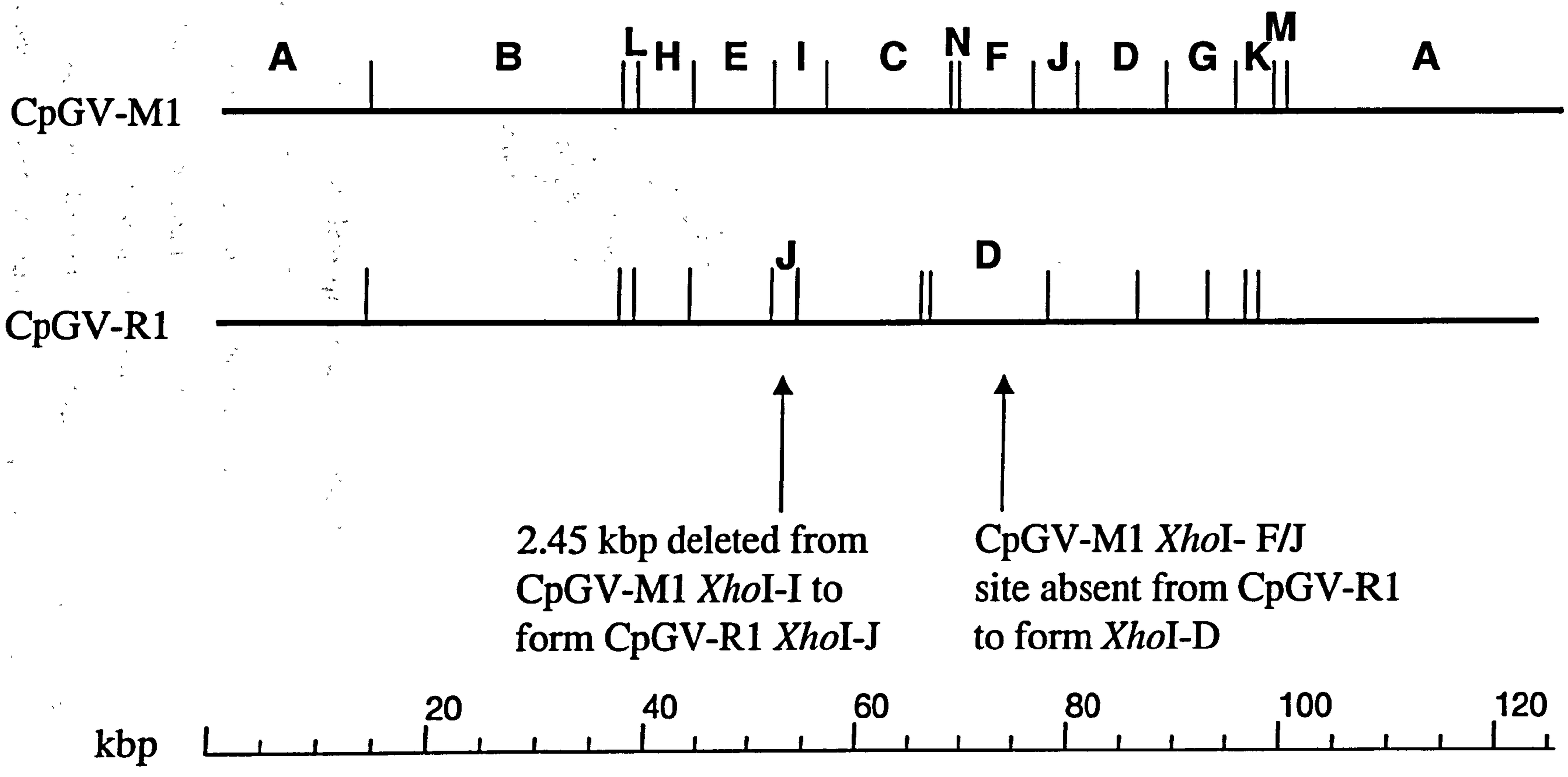
Two wells were selected for further work that had similar signal strengths as measured by spot diameters using the two probes. The medium that had been removed 6 d p.i. and 10 d p.i. was diluted across one plate of CpDW14R cells each. The remaining 5 µl cell material from the well was fed to fifth instar larvae. These larvae died of virus and OBs from the cadavers were purified and DNA extracted. A digest using *Xho*I was carried out to determine whether the virus was recombinant or simply CpGV-M1 contamination. The 2.45 kbp extra region in CpGV-M1 is within *Xho*I-I, so in CpGV-R1 this fragment is 2.53 kbp compared with *Xho*I-I in CpGV-M1 which is 4.98 kbp, Figure 6.4. This difference would not allow recombinant virus to be distinguished from CpGV-M1. However, there is a further difference between the *Xho*I profiles of CpGV-R1 and CpGV-M1 that is not due to the missing region. The *Xho*I site between CpGV-M1 *Xho*I-F and -J is missing in CpGV-R1 and so one larger band, *Xho*I-D is present in CpGV-R1, Figure 6.4.

The DNA from the infected insects was digested with *Xho*I and the restriction endonuclease profile appeared correct for a recombinant as it had a profile of CpGV-R1 with an extra 2.4 kbp in the corresponding fragment, Figure 6.5. The DNA was also digested with *Eco*R1 and an unexpected digest with submolar bands was obtained, which at the time was assumed to be an incomplete digestion, Figure 6.5.

Attempts were made to clone the virus from a larva *in vivo*. Larvae died 7-13 days post infection. Some were flaccid and translucent and did not lyse and others were large, white and looked like normal infected larvae. Larvae that died had their OBs crudely purified and DNA extracted. The DNA had the correct *Xho*I profile for the recombinant but still had the same *Eco*R1 profile which before was thought to be an incomplete digest, similar to Figure 6.5. This consisted of two extra faint submolar bands of approximately 7.5 kbp and 8.5 kbp, also *Eco*R1-D and -E were fainter, suggesting that the *Eco*R1 site between the two had disappeared, Figure 6.8. This would produce a band of about the same size as *Eco*R1-B and on these profiles *Eco*R1-B looked like it could have been a double band. The DNA from these insects was dotted onto a membrane and probed with CpGV-R1 and *Bgl*II-G. There was a lot

Figure 6.4

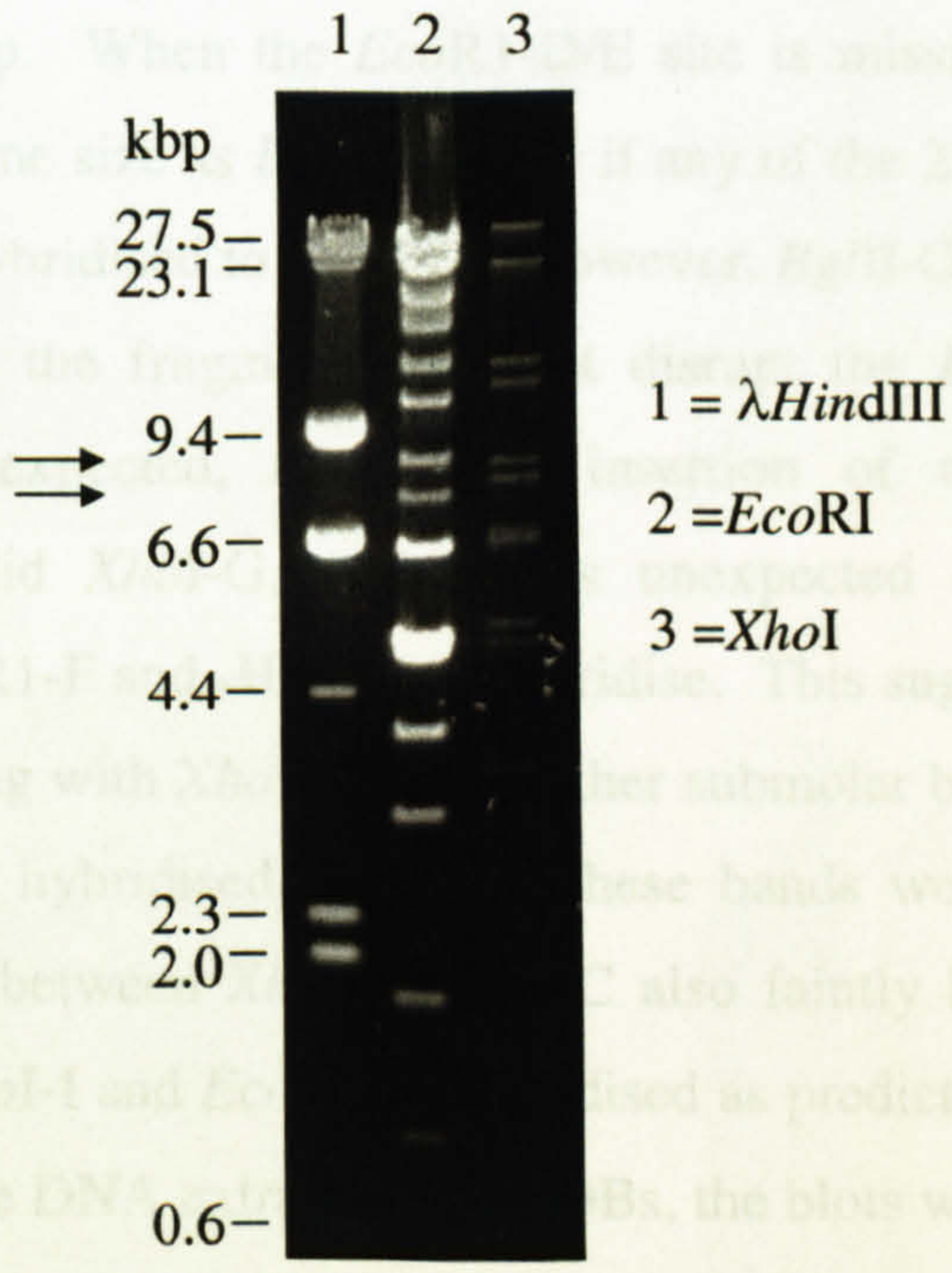
CpGV-M1 and CpGV-R1 *Xho*I restriction maps, indicating the differences between the two.



more CpGV-R1 hybridising than *Bgl*II-G, suggesting that CpGV-R1 was predominating (data not shown).

The plates produced during the third selection of recombinant virus were observed not to contain many OBs. The dot blots of the dilutions gave virtually identical strengths of signal using either of the probes, which suggested the recombinant was cloned.

Figure 6.5
OBs extracted from a larva, which had been infected with cells from a well infected with P4 virus. The restriction profiles show extra submolar bands (arrowed) in the *Eco*RI restriction endonuclease profile.



Fourth selection of recombinant virus (production of P6 virus)

To confirm that there were less OBs in the cells compared to normal infections, serial dilutions of P5 virus were used to inoculate 96 well plates seeded with CpDW14R cells. At 12 d p.i. there was an apparent cytopathic effect and cell lysis but no OBs were observed which was a function of the dilution of the inoculum. The DNA from these cells was not analysed by dot blot. Instead all the cells from each well across one row of the whole plate were fed to one fifth instar larva each. This was in the

event that there may have been a few OBs present that could infect the larvae. However, no larvae died from these infections.

Therefore, cells from *Bgl*II-G positive single wells infected with P2 and P3 virus were used to infect fifth instar larvae in an attempt to see if these would produce the *Eco*R1 profile with the submolar bands and the *Eco*R1 D/E site absent.

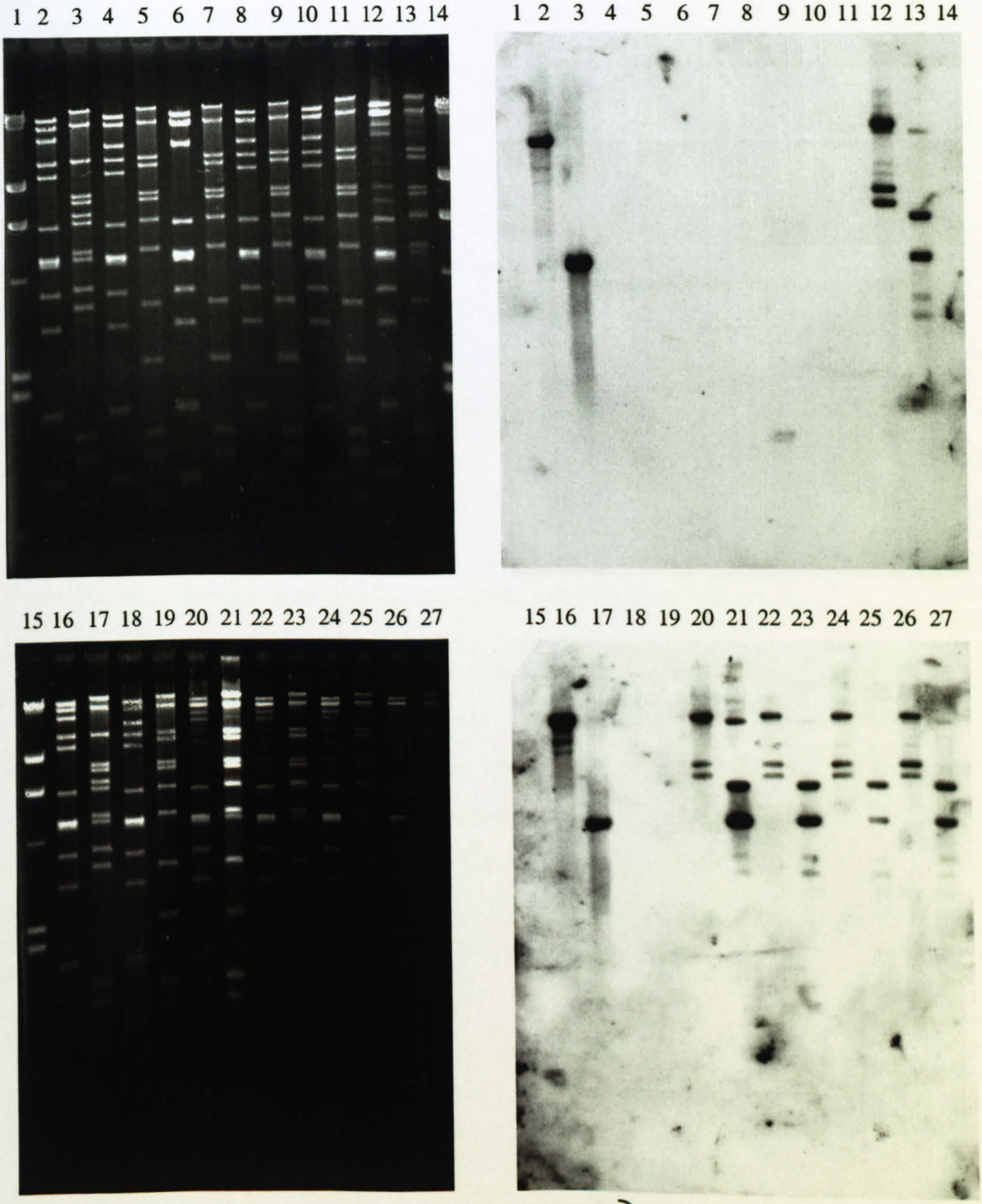
All the 'P3' larvae gave a recombinant profile with either *Eco*R1-D/E absent or very faint, Figure 6.6. They also had the two *Eco*R1 submolar bands. These profiles were Southern blotted and probed with *Bgl*II-G, Figure 6.6.

*Eco*R1-C hybridised as expected but so did the two faint *Eco*R1 submolar bands at 7.5 kbp and 8.5 kbp. When the *Eco*R1-D/E site is missing the two fragments form a fragment the same size as *Eco*R1-B and if any of the 2.45 kbp fragment had inserted, it would have hybridised to *Bgl*II-G. However, *Bgl*II-G did not hybridise to *Eco*RI-B, confirming that the fragment does not disrupt the *Eco*RI site. Fragment *Xho*I-I hybridised as expected, confirming insertion of the expected sequence here. However, so did *Xho*I-G, which was unexpected as the corresponding *Eco*R1 fragments, *Eco*R1-F and -H did not hybridise. This suggested the presence of another band comigrating with *Xho*I-G. Two other submolar bands that could hardly be seen on the gel also hybridised weakly. These bands were located around *Xho*I-J. A submolar band between *Xho*I-B and –C also faintly hybridised. In the CpGV-M1 control only *Xho*I-I and *Eco*R1-C hybridised as predicted. As so much *Bgl*II-G DNA hybridised to the DNA extracted from OBs, the blots were stripped and reprobed with pBSK+. This was in case any plasmid DNA had entered the viral genome. The pBSK+ plasmid was found to hybridise in several places, Figure 6.7. Within the *Eco*RI digests the submolar 8.5 kbp fragment hybridised, also two larger fragments and two other fragments faintly hybridised. Within the *Xho*I digest, a band comigrating with *Xho*I-G hybridised and three bands around the size of *Xho*I-J hybridised faintly.

The DNA from OBs of 'P2' infected larvae gave CpGV-R1 profiles. This suggested that the positive signal from the initial blot was due to recombinant DNA carry over during dot blotting. However, although this was probably also the case for insect C3,

Figure 6.6

Left panel: Gels of CpGV-M1 DNA, CpGV-R1 DNA and DNA of OBs from single larvae infected with cells from single wells of P2 and P3 infected plates.
Right panel: Autoradiographs of the gels probed with CpGV-M1 *Bgl*II-G fragment.



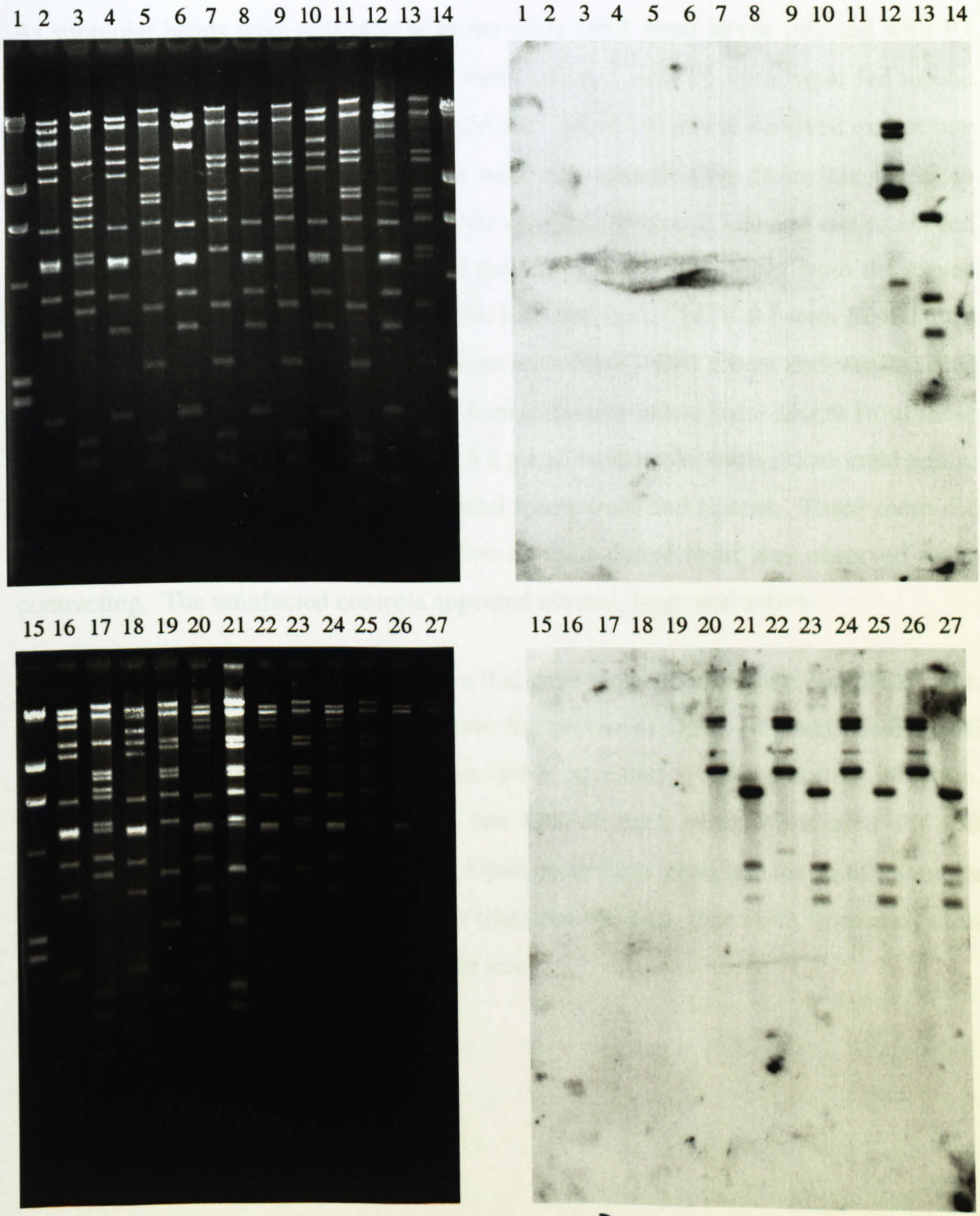
1, 14, 15 = λ*Hin*dIII
2, 16 = CpGV-M1 *Eco*RI
3, 17 = CpGV-M1 *Xho*I
4, 18 = CpGV-R1 EcoRI
5, 19 = CpGV-RI *Xho*I
6 = C3 *Eco*RI } P2
7 = C3 *Xho*I

8 = H3 *Eco*RI } P2
9 = H3 *Xho*I
10 = C1 *Eco*RI
11= C1 *Xho*I
12 = F1 *Eco*RI } P3
13 = F1 *Xho*I
20 = A5 *Eco*RI

21= A5 *Xho*I } P3
22 = A7 *Eco*RI
23 = A7 *Xho*I
24 = B4 *Eco*RI
25 = B4 *Xho*I
26 = H2 *Eco*RI
27 = H2 *Xho*I

Figure 6.7

Left panel: Gels of CpGV-M1 DNA, CpGV-R1 DNA and DNA of OBs from single larvae infected with cells from single wells of P2 and P3 infected plates.
Right panel: Autoradiographs of the gels probed with pBSK+ DNA.



1, 14, 15 = λ*Hin*dIII
2, 16 = CpGV-M1 *Eco*RI
3, 17 = CpGV-M1 *Xho*I
4, 18 = CpGV-R1 EcoRI
5, 19 = CpGV-RI *Xho*I
6 = C3 *Eco*RI } P2
7 = C3 *Xho*I

8 = H3 *Eco*RI } P2
9 = H3 *Xho*I
10 = C1 *Eco*RI
11= C1 *Xho*I
12 = F1 *Eco*RI } P3
13 = F1 *Xho*I
20 = A5 *Eco*RI

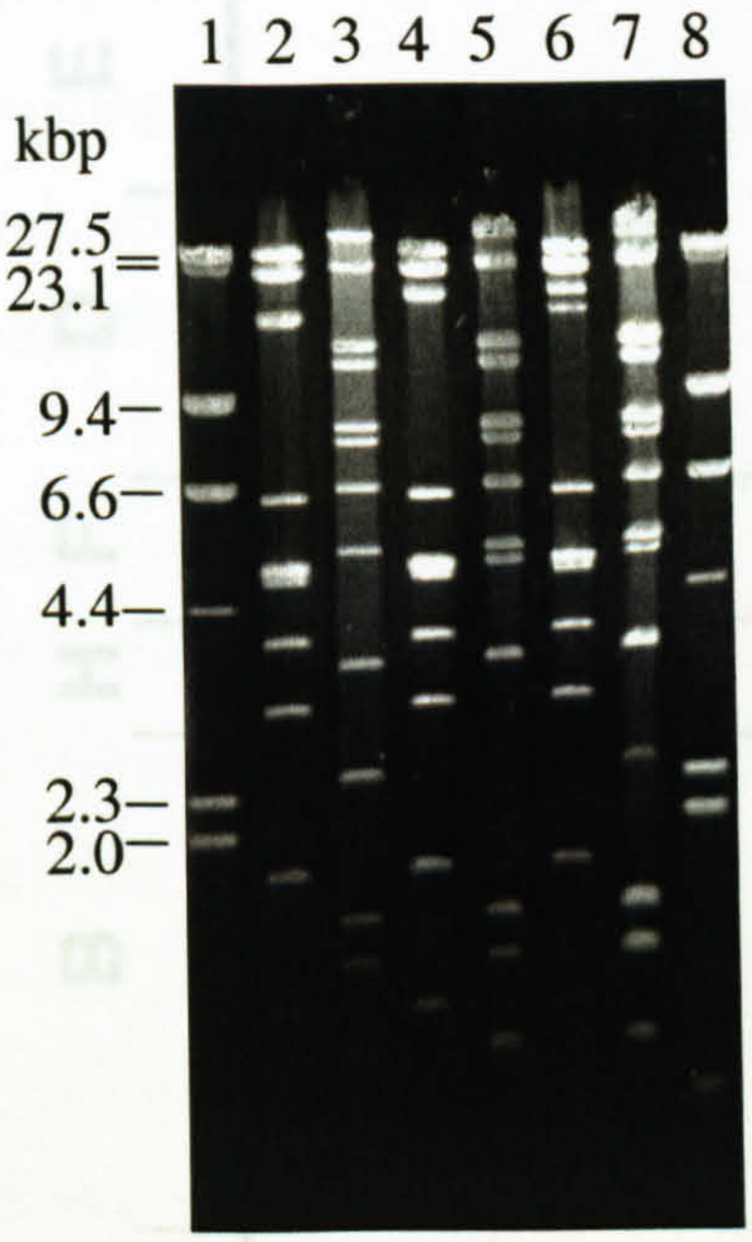
21= A5 *Xho*I } P3
22 = A7 *Eco*RI
23 = A7 *Xho*I
24 = B4 *Eco*RI
25 = B4 *Xho*I
26 = H2 *Eco*RI
27 = H2 *Xho*I

there appeared to be a CpGV-R1 with the *Eco*R1-D and -E missing here also, Figure 6.6. This profile contained no submolar bands. Thus although this *Eco*RI restriction profile change was associated with the presence of the CpGV-R1/CpGV-M1 recombinant it was not generated as part of the recombination process.

As submolar bands were still present in the virus DNA from larvae infected with P3 virus, half of the cells from each of 12 wells infected with P5 virus were fed to one fifth instar larvae each to see if they would die. All of the larvae survived except one which died flaccid at 11 d p.i. There were not enough OBs from this insect to perform a DNA extraction so the OBs were counted (section 2.7.2) and one or ten fed to fifth instar larvae. Three larvae died quickly at 7-8 d p.i. Virus from the larvae was OB-purified and DNA extracted. One had died from CpGV-R1 with *Eco*R1-D/E site absent. One had died from recombinant with *Eco*R1-D/E absent and one had died from a mix of the two, Figure 6.8. The faint submolar bands were absent from these profiles. A further five larvae died 14-15 d p.i. These larvae looked abnormal again; they had patchy melanisation and appeared transparent and flaccid. Three more did not respond to stimuli but were still alive as their dorsal heart was observed to be contracting. The uninfected controls appeared normal, large and active.

One and ten OBs obtained from the larva that gave the profile of the recombinant with *Eco*R1-D/E absent and the larva that gave the profile of CpGV-R1 with *Eco*R1-D/E absent were fed to further larvae. These larvae appeared to have a normal infection. The first five larvae that died from one OB of each virus were used for OB purification and DNA was extracted. Upon restriction analysis, the faint submolar bands were still absent from the profiles (data not shown). One larva from each virus infection was used to clone the viruses *in vivo*.

Figure 6.8
Restriction profiles of DNA from OBs recovered from three larvae that died 7-8 d p.i. Larva 1 has a profile of CpGV-R1 with *Eco*RI-D/E site absent. Larva 2 has a profile of a CpGV-R1/*Sal*I-F recombinant with *Eco*RI-D/E site absent and larva 3 has a mix of the virus from 1 and 2.



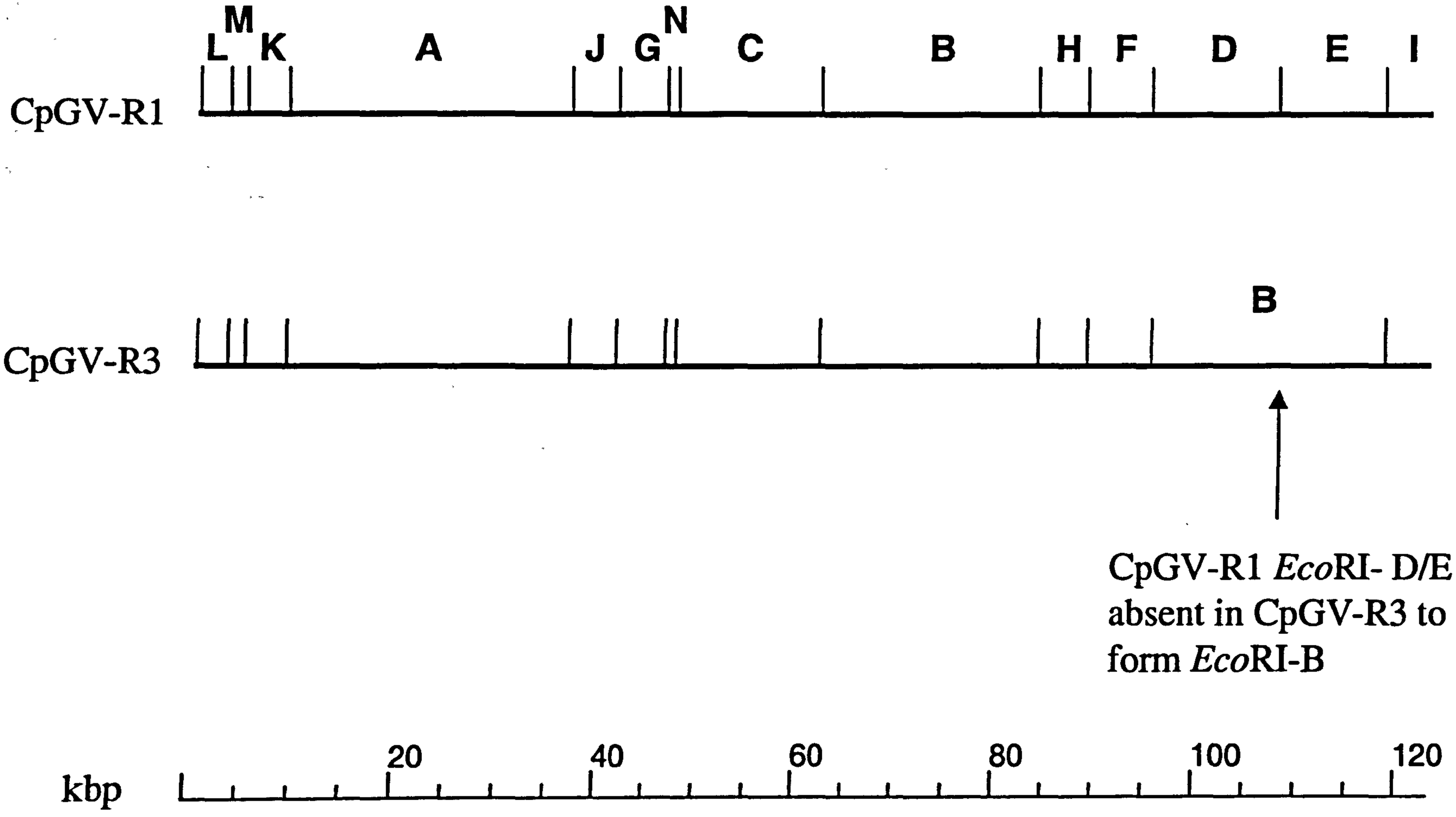
6.2.2 The origin of CpGV-R1 with *Eco*R1-D/E absent (CpGV-R3)

The CpGV-R1 with *Eco*R1-D/E absent was named CpGV-R3, Figure 6.9, and the recombinant, CpGV-R3^R. There were three possible explanations for the appearance of CpGV-R3.

1) There may have been a small proportion of CpGV-R3 in the initial CpGV-R1 stock. The profiles for CpGV-R1 appeared correct but as the *Eco*RI-D and -E formed a band about the same size as *Eco*R1-B it would have been difficult to tell from an *Eco*R1 profile, if CpGV-R3 was present, as no extra bands would have been apparent. *Eco*R1 -D and -E did not appear fainter than other bands, so if CpGV-R3 was there, it must have been a very small proportion. All the other profiles using different restriction enzymes were the same as CpGV-R1. The plasmid *Sal*I-F must then have recombined with CpGV-R3 to form CpGV-R3^R, since this virus also does not have *Eco*R1 -D and –E as separate fragments.

Figure 6.9

CpGV-R1 and CpGV-R3 *Eco*RI restriction maps, indicating the difference between the two.



2) Part of *Sal*I-F may have disrupted the *Eco*RI site of CpGV-R1 by recombination and formed CpGV-R3, which then recombined again with *Sal*I-F to form CpGV-R3^{R}. However, CpGV-M1 *Bgl*II-G did not hybridise to CpGV-R3.

3) By passing CpGV-R1 through cells, a cell culture variant may have been formed, with the *Eco*R1-D/E site absent. This variant then recombined with *Sal*I-F to form R3^{R}.

These hypotheses were investigated by firstly feeding fifth instar larvae cells with a low level of infection from wells of the original P2 plate of control cells infected with CpGV-R1 P1 virus (not transfected with *Sal*I-F). The OBs from individual larvae that died, were purified and then DNA extracted (sections 2.7.1.2 and 2.7.4). The DNA was digested with *Eco*RI. The profiles were all of CpGV-R1 and not CpGV-R3 (data not shown). The original CpGV-R1 was also fed in low doses (12% death) to 100 fifth instar larvae and single insect OB purifications and DNA extractions were performed. All larvae died of CpGV-R1 (data not shown). Therefore, it appeared from these investigations that if CpGV-R3 was present in the original stock of CpGV-R1 then it was at a very low level.

A 805 bp region surrounding the *Eco*R1 D/E site was amplified by PCR from all four of the genotypes and sequenced (section 2.4) using the following primers:

Upper primer	R1Eco1:	5' TTTGCGCCCGAGATCGAAGAGAAG 3'
Lower primer	R1Eco2:	5' TAACGGTGATGAGGAGCAGGAGTA 3'

A length of 657 bp of the sequences is shown in Figure 6.10. In CpGV-R3 and CpGV-R3^{R}, the *Eco*R1 site had been disrupted by a base pair substitution. This modified the *Eco*R1 site GAATTC into GAGTTC with the base pair substitution at position 356. There are other differences between CpGV-R3 and CpGV-R1. CpGV-R3 has a C instead of a T at position 214 and 481 and a C instead of a G at position 532. There are no differences between CpGV-R1 and CpGV-M1 in this region, suggesting that CpGV-R3 is a completely different genotype to CpGV-R1 and CpGV-

Figure 6.10

Multiple alignments of the nucleotide sequence of 657 bp of the *Eco*RI-D/E restriction site area of the four CpGV genotypes. Regions of identity are shaded pink. The *Eco*RI site is boxed.

```
                10        20        30        40        50
1   CACACATGATGAGGCCAACACAATCAACAACGAGTGCTACAATTTTGTGG  M1
1   CACACATGATGAGGCCAACACAATCAACAACGAGTGCTACAATTTTGTGG  R1
1   CACACATGATGAGGCCAACACAATCAACAACGAGTGCTACAATTTTGTGG  R3R
1   CACACATGATGAGGCCAACACAATCAACAACGAGTGCTACAATTTTGTGG  R3

          60        70        80        90       100
51  TGAACGAGGCGGAAACCACGTTAAAGTGCACAACAAAACTGGTGCCGTGG  M1
51  TGAACGAGGCGGAAACCACGTTAAAGTGCACAACAAAACTGGTGCCGTGG  R1
51  TGAACGAGGCGGAAACCACGTTAAAGTGCACAACAAAACTGGTGCCGTGG  R3R
51  TGAACGAGGCGGAAACCACGTTAAAGTGCACAACAAAACTGGTGCCGTGG  R3

         110       120       130       140       150
101 TCGTACAAGGTCAATGTGTTCAATGCGCAACAAGAGAGGTTGATATTTAA  M1
101 TCGTACAAGGTCAATGTGTTCAATGCGCAACAAGAGAGGTTGATATTTAA  R1
101 TCGTACAAGGTCAATGTGTTCAATGCGCAACAAGAGAGGTTGATATTTAA  R3R
101 TCGTACAAGGTCAATGTGTTCAATGCGCAACAAGAGAGGTTGATATTTAA  R3

         160       170       180       190       200
151 GCAAGAGTTGCAAGTGTGTCCGGGTACCAGGGTGATGGTGACACACACCA  M1
151 GCAAGAGTTGCAAGTGTGTCCGGGTACCAGGGTGATGGTGACACACACCA  R1
151 GCAAGAGTTGCAAGTGTGTCCGGGTACCAGGGTGATGGTGACACACACCA  R3R
151 GCAAGAGTTGCAAGTGTGTCCGGGTACCAGGGTGATGGTGACACACACCA  R3

         210       220       230       240       250
201 CTCAACATTTTTGTAACGGTGACACGGGCATTATAGAGTACATCACTGAT  M1
201 CTCAACATTTTTGTAACGGTGACACGGGCATTATAGAGTACATCACTGAT  R1
201 CTCAACATTTTTGCAACGGTGACACGGGCATTATAGAGTACATCACTGAT  R3R
201 CTCAACATTTTTGCAACGGTGACACGGGCATTATAGAGTACATCACTGAT  R3

         260       270       280       290       300
251 ATGGGTGTGTACATTAGGAGGGAGCACGACAATTCGCTGCAAATATTGGG  M1
251 ATGGGTGTGTACATTAGGAGGGAGCACGACAATTCGCTGCAAATATTGGG  R1
251 ATGGGTGTGTACATTAGGAGGGAGCACGACAATTCGCTGCAAATATTGGG  R3R
251 ATGGGTGTGTACATTAGGAGGGAGCACGACAATTCGCTGCAAATATTGGG  R3

         310       320       330       340       350
301 ACCGATCACGCTACACTTTAGTTCTAGTCGCGGCTACATTAAAGCGGTGA  M1
301 ACCGATCACGCTACACTTTAGTTCTAGTCGCGGCTACATTAAAGCGGTGA  R1
301 ACCGATCACGCTACACTTTAGTTCTAGTCGCGGCTACATTAAAGCGGTGA  R3R
301 ACCGATCACGCTACACTTTAGTTCTAGTCGCGGCTACATTAAAGCGGTGA  R3

         360       370       380       390       400
351 CAGGAATTCCTCTCAACTATGGATGGGCGGTGACCATACATAAAGCGCAA  M1
351 CAGGAATTCCTCTCAACTATGGATGGGCGGTGACCATACATAAAGCGCAA  R1
351 CAGGAGTTCCTCTCAACTATGGATGGGCGGTGACCATACATAAAGCGCAA  R3R
351 CAGGAGTTCCTCTCAACTATGGATGGGCGGTGACCATACATAAAGCGCAA  R3
```

```
                410        420        430        440        450
401 GGTATGACCATCAAAAATCTAATAGTGTACCCCAAGTGTTTGTTTGCACC  M1
401 GGTATGACCATCAAAAATCTAATAGTGTACCCCAAGTGTTTGTTTGCACC  R1
401 GGTATGACCATCAAAAATCTAATAGTGTACCCCAAGTGTTTGTTTGCACC  R3R
401 GGTATGACCATCAAAAATCTAATAGTGTACCCCAAGTGTTTGTTTGCACC  R3

                460        470        480        490        500
451 GGGTCAAGCGTACGTGGCGCTAAGCAGATGTACCCACTCGAAAGGATTGA  M1
451 GGGTCAAGCGTACGTGGCGCTAAGCAGATGTACCCACTCGAAAGGATTGA  R1
451 GGGTCAAGCGTACGTGGCGCTAAGCAGATGCACCCACTCGAAAGGATTGA  R3R
451 GGGTCAAGCGTACGTGGCGCTAAGCAGATGCACCCACTCGAAAGGATTGA  R3

                510        520        530        540        550
501 AATTATCGTGTCGTATACCCGACGAGGGTGTGAAGGATATGAGCTTTATT  M1
501 AATTATCGTGTCGTATACCCGACGAGGGTGTGAAGGATATGAGCTTTATT  R1
501 AATTATCGTGTCGTATACCCGACGAGGGTGTCAAGGATATGAGCTTTATT  R3R
501 AATTATCGTGTCGTATACCCGACGAGGGTGTCAAGGATATGAGCTTTATT  R3

                560        570        580        590        600
551 ACCGAGGTGTATAACAGTATGGAAAAATGGTGTTAGTGATAGACGGTTTA  M1
551 ACCGAGGTGTATAACAGTATGGAAAAATGGTGTTAGTGATAGACGGTTTA  R1
551 ACCGAGGTGTATAACAGTATGGAAAAATGGTGTTAGTGATAGACGGTTTA  R3R
551 ACCGAGGTGTATAACAGTATGGAAAAATGGTGTTAGTGATAGACGGTTTA  R3

                610        620        630        640        650
601 TGTATCGTGATCGTTTTTATTCAATAAACACATACACAGCAGCAGCAACA  M1
601 TGTATCGTGATCGTTTTTATTCAATAAACACATACACAGCAGCAGCAACA  R1
601 TGTATCGTGATCGTTTTTATTCAATAAACACATACACAGCAGCAGCAACA  R3R
601 TGTATCGTGATCGTTTTTATTCAATAAACACATACACAGCAGCAGCAACA  R3

651 TTTTATT  M1
651 TTTTATT  R1
651 TTTTATT  R3R
651 TTTTATT  R3
```

M1. CpGV-R3^{R} is the same in this region as CpGV-R3 suggesting that CpGV-R3 recombined with *Sal*I-F to form CpGV-R3^{R}.

The base pair differences between the genotypes are within an ORF with similarities to LdMNPV Helicase-2 (Kuzio, *et al.*, 1999). However, three of the four differences result in the same amino acid and the other one changes the amino acid to a similar one. Therefore, these differences are unlikely to affect the virus.

It is unlikely that a cell culture variant could be formed that changed a base pair every 150-200 bp after just two passages in cell culture. The sequence data clearly also excludes the possibility that loss of the *Eco*RI site resulted from recombination with *Sal*I-F DNA. Consequently, CpGV-R3 most likely originated as a contaminant of the CpGV-R1 stock, which recombined with *Sal*I-F to form CpGV-R3^{R}.

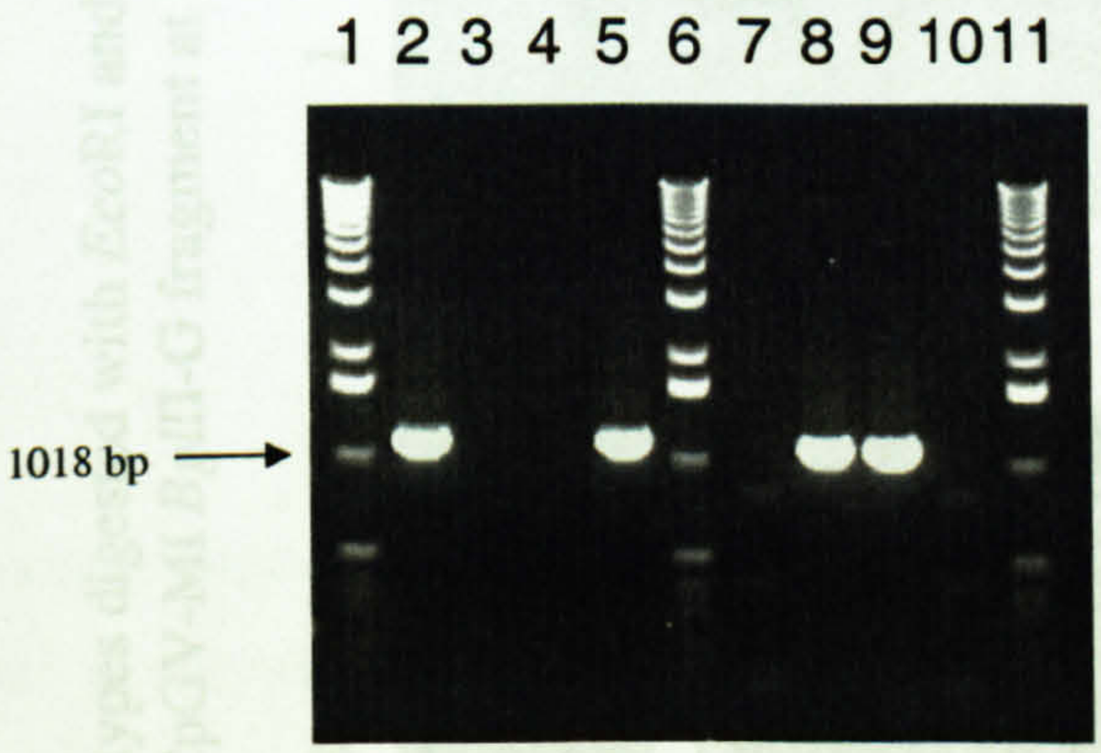
6.2.3 *In vivo* cloning of CpGV-R3 and CpGV-R3^{R}

The viruses CpGV-R1, CpGV-R3 and CpGV-R3^{R} were cloned *in vivo* by feeding low doses to fifth instar larvae (section 2.6.5). After three rounds of the progeny virus appearing homogeneous, the DNA was checked by PCR using the REPT primers, Figure 6.11. Only CpGV-R1 and CpGV-R3 gave products; these were of the expected size. The amplification product expected from CpGV-M1 and CpGV-R3^{R} were not observed under these conditions. During these studies, CpGV *Sal*I-F was also being re-sequenced for the CpGV-M1 sequencing project (D. Winstanley and D. O'Reilly, personal communication). This allowed PCR primers to be designed to amplify DNA within the extra CpGV-M1 DNA. The primers are shown below. These primers amplified a fragment of 1117 bp from CpGV-M1 and CpGV-R3^{R}, Figure 6.11. No product was amplified from CpGV-R1 or CpGV-R3. This allowed CpGV-R1 and CpGV-R3 to be checked for the absence of CpGV-M1 and CpGV-R3^{R}. The original CpGV-R1 DNA was also checked and no CpGV-M1 sized product was produced. The result of the PCR amplification of each viral DNA was as predicted for each virus, indicating that the virus was cloned.

Upper primer	M1insertb 1:	5' TTTCGGCGTGCAATCGGTAAGTGT 3'
Lower primer	M1insertb 2:	5' GCGCGGTGCTAGGAGGGAAGTC 3'

Restriction digests were performed on purified viral DNA of each genotype, using the enzymes *Eco*RI and *Xho*I. The gels were blotted and probed with *Bgl*II-G to check for the presence or absence of the fragment, Figure 6.12. CpGV-R3^R produced single hybridising fragments of the correct size with each enzyme. The viruses were then amplified in fifth instar larvae by feeding larvae with 1000 OBs each which is an approximate LD_{100}. The progeny virus was checked again and each found to be the correct genotype (data not shown).

Figure 6.11
PCR products from the four CpGV genotypes using Rept primers or M1insertb primers to amplify the DNA



1+6+11 = Kb ladder

2 = CpGV-R1 Rept primers

3 = CpGV-M1 Rept primers

4 = CpGV-R3^R Rept primers

5 = CpGV-R3 Rept primers

7 = CpGV-R1 M1insertb primers

8 = CpGV-M1 M1insertb primers

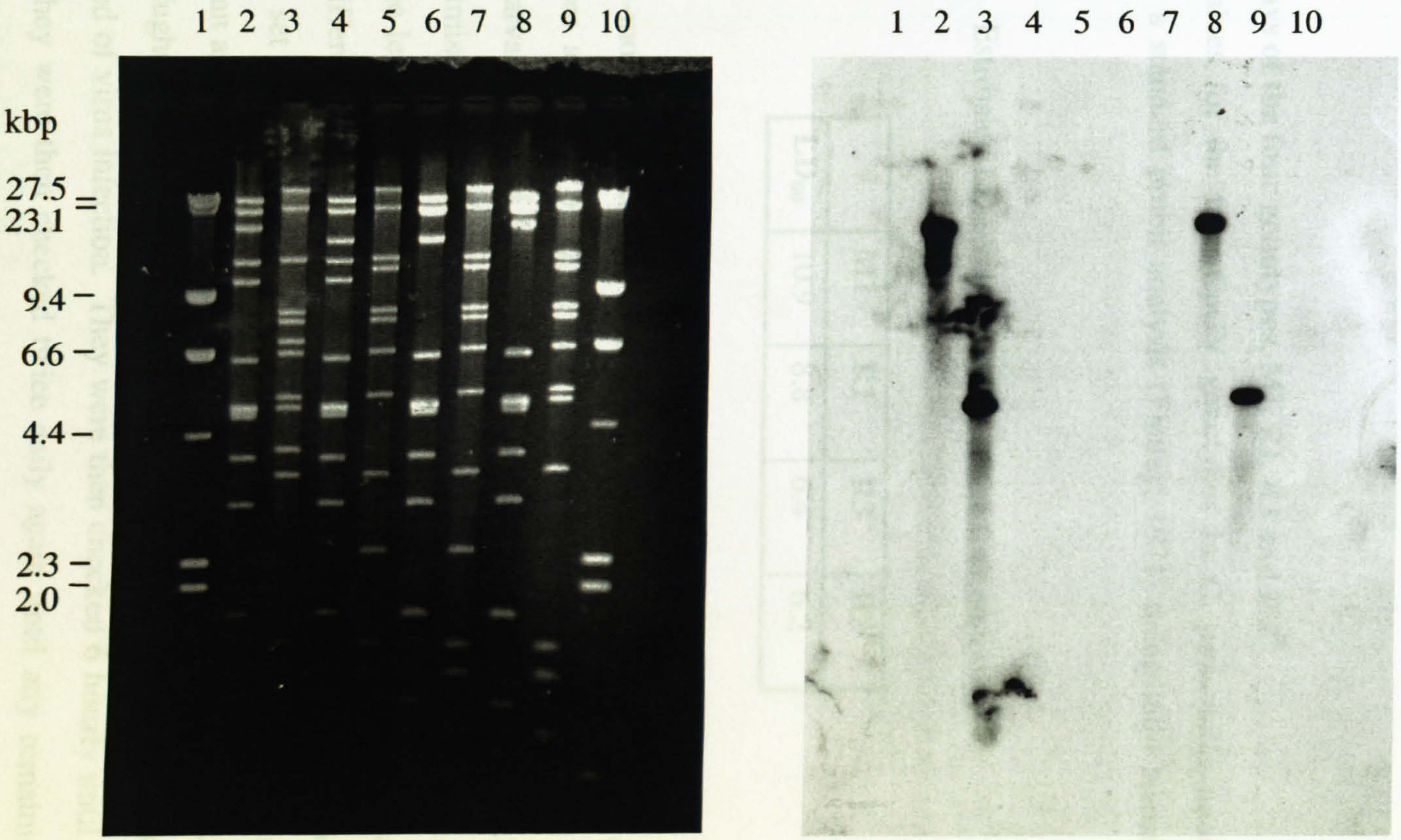
9 = CpGV-R3^R M1insertb primers

10 = CpGV-R3 M1insertb primers

Restriction digests were performed on all of the genotypes to try and determine if there were other differences between them, based on their physical map. Only the expected differences, due to the 2.45 kbp insertion, were observed using the enzymes that have been mapped for CpGV-M1. These enzymes are *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Sac*I, *Sal*I, *Sma*I and *Xho*I. They showed no other differences between CpGV-R1, CpGV-R3 and CpGV-R3^R (data not shown). The enzyme *Pst*I gave a different profile than expected for CpGV-R1. The fragment *Pst*I-A of CpGV-M1 (27.9 kbp) was cleaved at two additional sites in CpGV-R1 into approximately

Figure 6.12

Left panel: 0.7 % agarose gel of the four CpGV genotypes digested with *Eco*RI and *Xho*I
Right panel: Autoradiograph of the gel probed with CpGV-MI *Bgl*II-G fragment at high stringency (65°C)



1 + 10 = λ*Hin*dIII
2 = CpGV-M1 digested with *Eco*RI
3 = CpGV-M1 digested with *Xho*I
4 = CpGV-R1 digested with *Eco*RI
5 = CpGV-R1 digested with *Xho*I
6 = CpGV-R3 digested with *Eco*RI
7= CpGV-R3 digested with *Xho*I
8 = CpGV-R3^R digested with *Eco*RI
9= CpGV-R3^R digested with *Xho*I

12.5 kbp, 8.4 kbp and 7.0 kbp fragments. The order of these fragments has not yet been determined. CpGV-R3 and CpGV-R3^R also have these additional sites. Therefore, CpGV-R3 and CpGV-R1 have the same restriction profiles using the restriction enzymes named above, apart from the deleted *Eco*RI site in CpGV-R3. The genotypes CpGV-R3 and CpGV-R3^R appear identical except for the 2.45 kbp CpGV-M1 insert in *Sal*I-F.

6.2.4 Bioassays of the four genotypes, MI, RI, R3 and R3^R

The LD_{80} values for the four CpGV genotypes in *C. pomonella* neonates were estimated by a standard probit analysis (Finney, 1971) using logit transformations, Table 6.1.

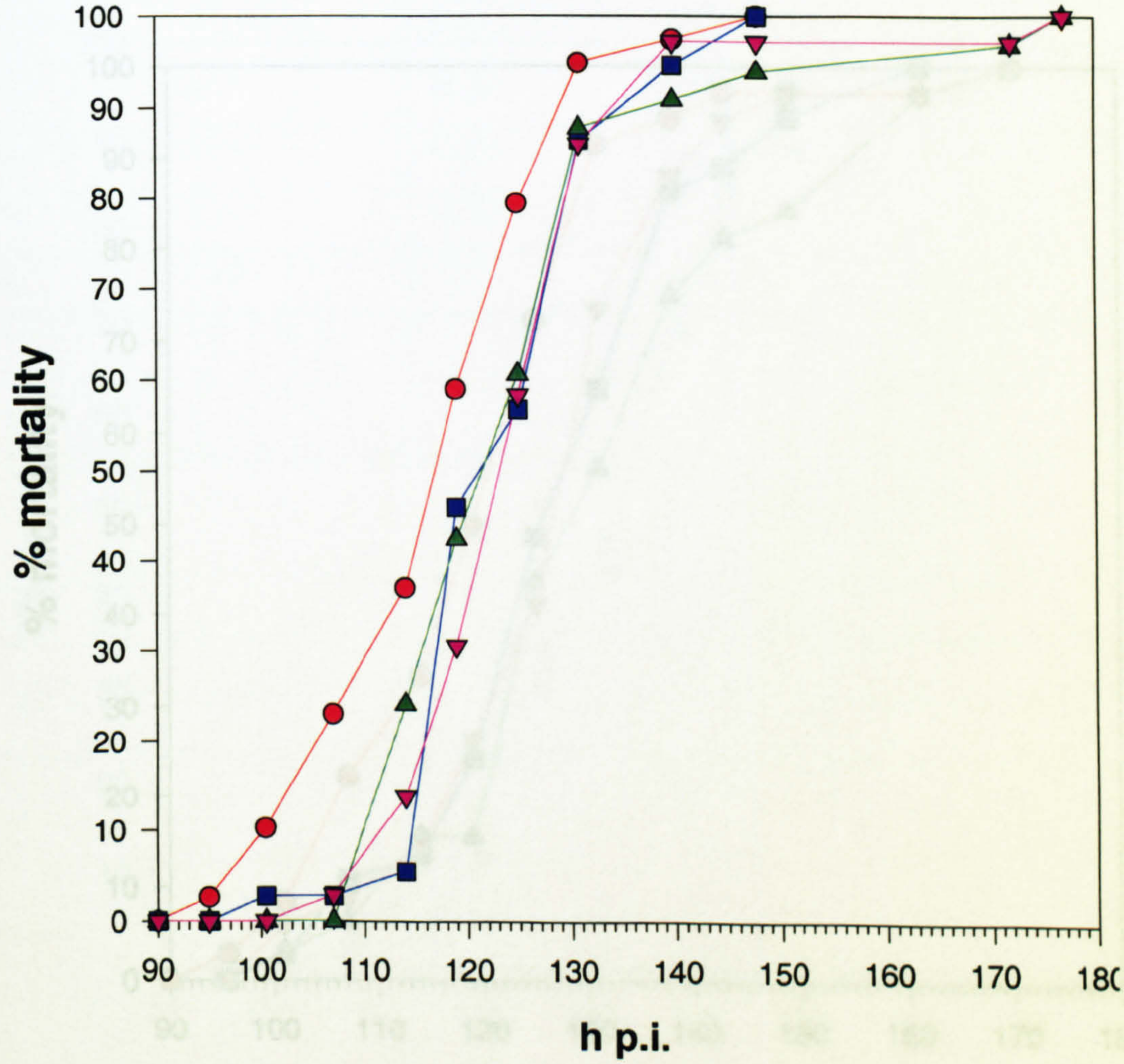
Table 6.1 Estimated LD_{80} values in OBs of the four genotypes.

	M1	R1	R3	R3^R
LD_{80}	10.9	8.8	6.4	6.2

Neonate time-mortality bioassays were then performed in triplicate using each of the four genotypes at a dose of LD_{80}. Forty-eight neonates were infected with each genotype. Larvae that died from handling were excluded from the calculations. In order to minimise any variation caused by differences in the length of starvation of larvae and the length of time it took to set up each virus bioassay, the sequence in which the different virus genotypes were given to larvae was randomised. The experimental set up is such that all of the larvae receive a particular dose within a short time span and are held on non-contaminated diet throughout the remaining time of the test (Hughes *et al.*, 1997). Larvae were checked twice daily for death until the first larva died of virus infection. They were then checked 6 hourly until about 80% had died. They were then checked twice daily again until any remaining infected larvae died. Raw data collected from the bioassays are shown in Appendix 9. Time-mortality graphs for each of the three bioassays are shown in Figures 6.13-6.15. The actual percentage of larvae that died from each genotype in each bioassay are shown

Figure 6.13

Bioassay 1 mortality curves for the four CpGV genotypes infected with an LD_{80} dose. Mortality is shown as a percentage of the larvae that responded to virus infection.



= CpGV-M1

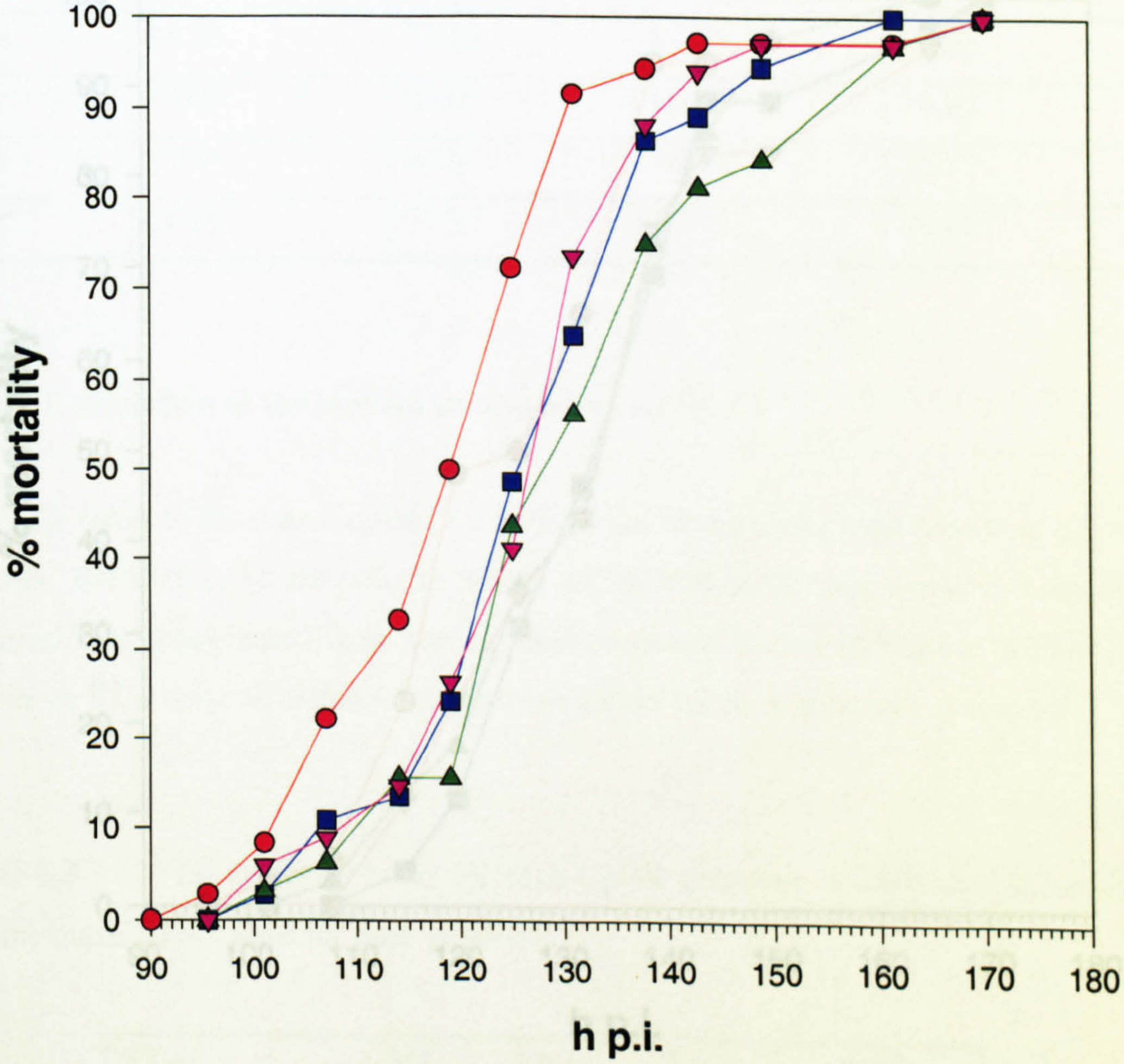
= CpGV-R1

= CpGV-R3

= CpGV-$R3^R$

Figure 6.14

Bioassay 2 mortality curves for the four CpGV genotypes infected with an LD_{80} dose. Mortality is shown as a percentage of the larvae that responded to virus infection.



= CpGV-M1

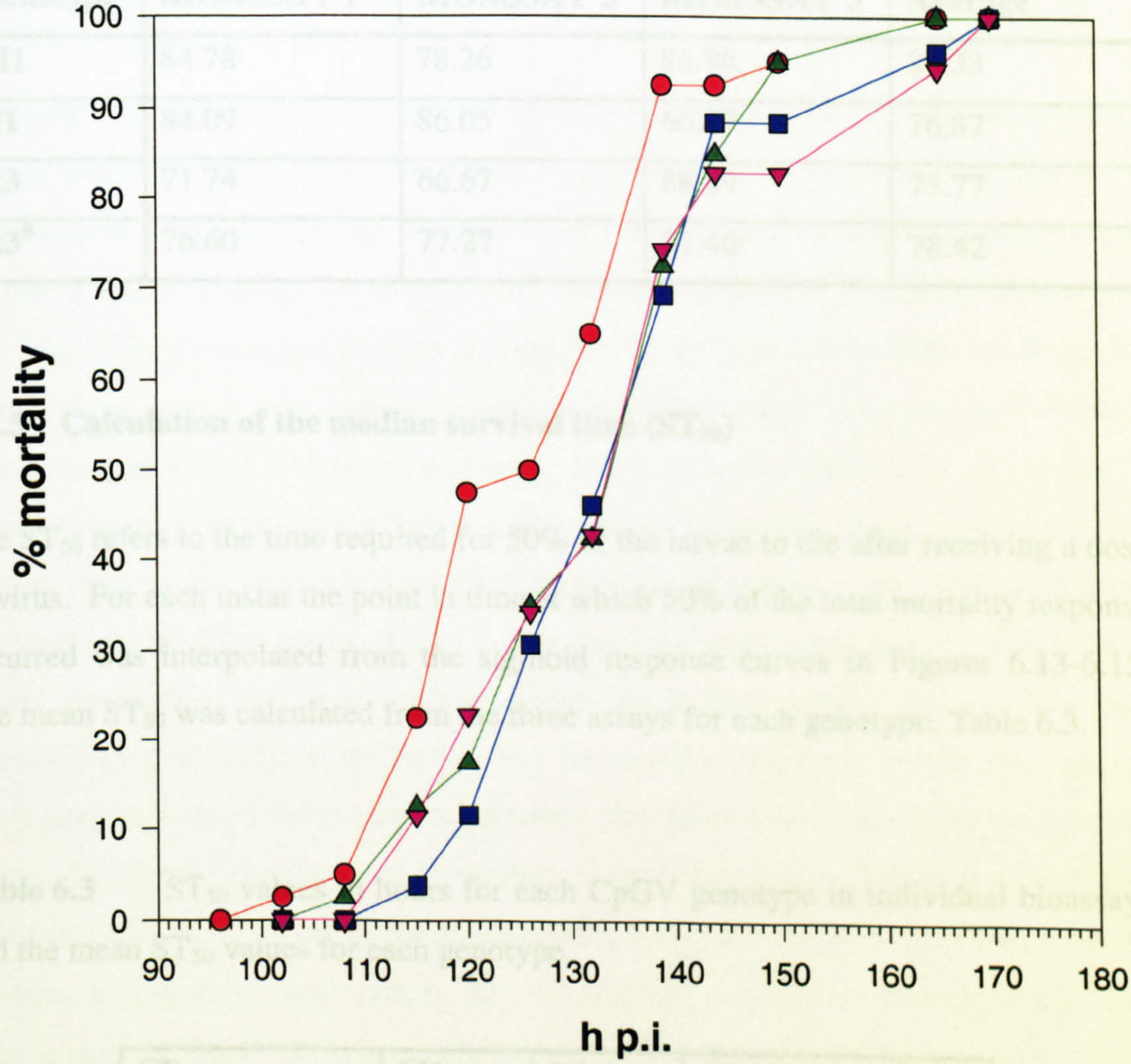
= CpGV-R1

= CpGV-R3

= CpGV-R3^R

Figure 6.15

Bioassay 3 mortality curves for the four CpGV genotypes infected with an LD_{80} dose. Mortality is shown as a percentage of the larvae that responded to virus infection.



= CpGV-M1

= CpGV-R1

= CpGV-R3

= CpGV-R3^R

in Table 6.2. However, the graphs represent the total number of larvae that died of virus infection as 100% mortality for clarity.

Table 6.2 Percentage mortality of neonate *C. pomonella* larvae for each CpGV genotype in each bioassay

Genotype	BIOASSAY 1	BIOASSAY 2	BIOASSAY 3	Average
M1	84.78	78.26	86.96	83.33
R1	84.09	86.05	60.47	76.87
R3	71.74	66.67	88.89	75.77
$R3^R$	76.60	77.27	81.40	78.42

6.2.5 Calculation of the median survival time (ST_{50})

The ST_{50} refers to the time required for 50% of the larvae to die after receiving a dose of virus. For each instar the point in time at which 50% of the total mortality response occurred was interpolated from the sigmoid response curves in Figures 6.13-6.15. The mean ST_{50} was calculated from the three assays for each genotype, Table 6.3.

Table 6.3 ST_{50} values in hours for each CpGV genotype in individual bioassays and the mean ST_{50} values for each genotype.

ST_{50}	M1	R1	R3	$R3^R$
BIOASSAY 1	117.0	121.3	121.0	122.2
BIOASSAY 2	119.0	125.7	128.0	125.6
BIOASSAY 3	126.0	133.0	134.3	133.1
Mean	120.7	126.7	127.8	127.0

Mean ST_{50} values for all genotypes were analysed using analysis of variance (Sokal and Rohlf, 1995) using a randomised complete block model (bioassays as blocks). For genotypes, a contrast (comparison) was made between the Mexican and the mean of the Russian genotypes. The principal findings to emerge from these analyses were that the ST_{50} of CpGV-M1 was significantly lower ($F_{1,6} = 92.99$ $p < 0.01$) than the ST_{50} of the Russian genotypes. In addition, the ST_{50} for the three Russian isolates did not differ significantly from one another.

Although the bioassays were repeated as consistently as possible with all bioassays taking place at exactly 25°C, the larvae progressively took longer to kill, Table 6.4. The second bioassay was, on average 4.2 hours slower than the first and the third bioassay was on average 7.0 hours slower than the second and 11.2 hours slower than the first. The viruses had not lost any infectivity as LD_{80} values were not declining. When these values were compared with values from other bioassays performed on neonates with CpGV-M1 in the previous year, they were found to be on average 13 hours slower. Larvae were infected with either an LD_{80} dose of the virus stock used in this study or the virus stock used in the previous bioassays. They were both found to have similar ST_{50} values of 125 hours and 126 hours respectively. This was 16 hours slower than the same bioassay performed in December of the previous year. This shows that although the larvae may be reared at the same temperature and the bioassays performed at the same temperature throughout the year, the larvae may still respond differently to the virus depending on the time of year. The general fecundity of the *C. pomonella* larvae in the insect rearing unit often drops in the winter even in a control temperature environment (D. Winstanley, personal communication). The larvae may be 'fitter' in summer months and therefore less susceptible to disease.

Time-mortality bioassays would need to be performed at different times in the year to see if the speed of kill increases again, as bioassays have only been performed where the speed of kill has decreased.

Table 6.4

ST_{50} values in hours for bioassays of *C. pomonella* neonate larvae infected with an LD_{80} dose of CpGV-M1, performed at different times of the year.

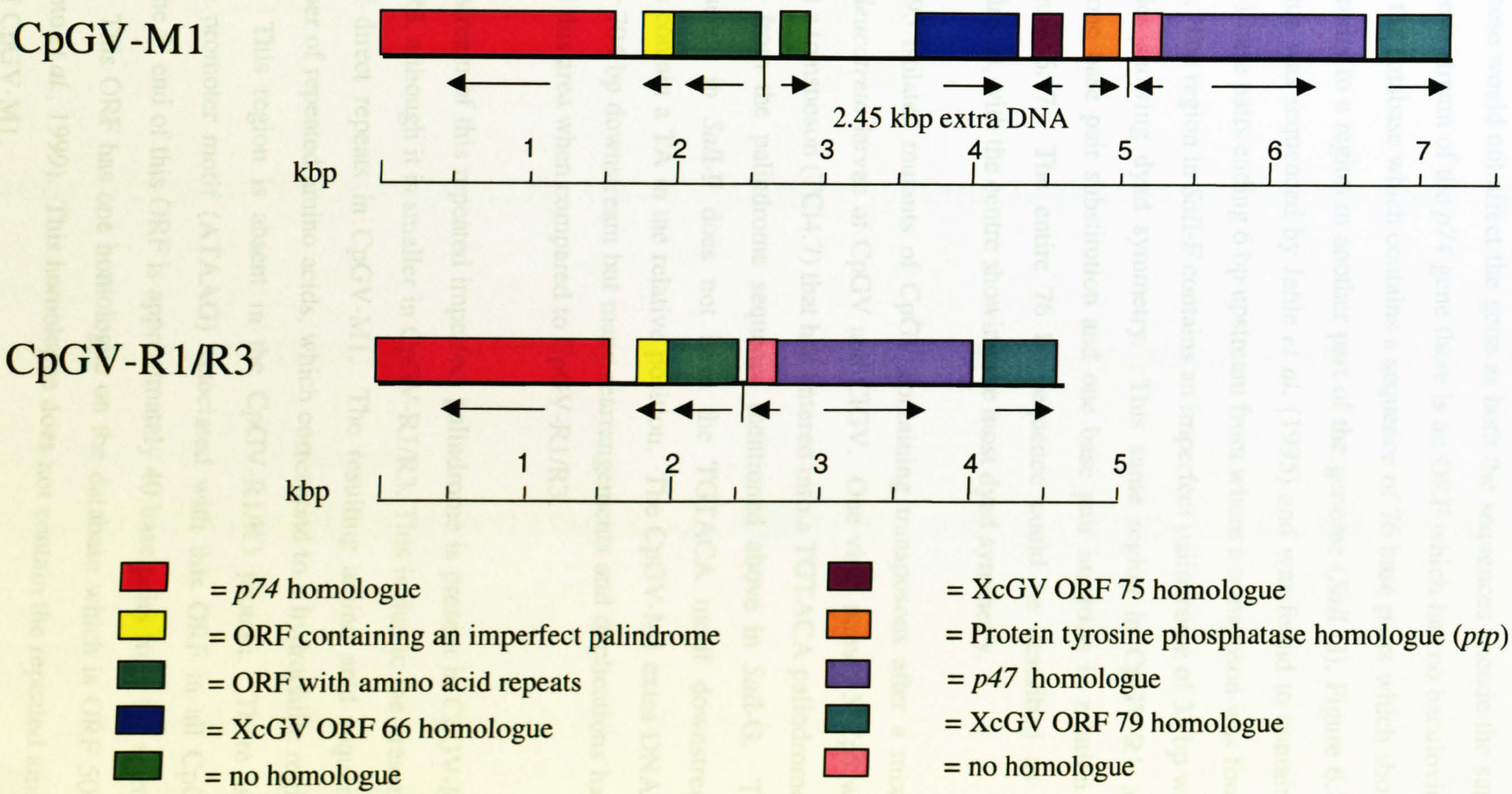
BIOASSAY	MONTH PERFORMED	ST_{50}
Previous Bioassay	December 1998	110
Bioassay 1	June 1999	117
Bioassay 2	July 1999	119
Bioassay 3	September 1999	126

6.2.6 Sequencing within the *Sal*I-F fragment

Recently, the *Sal*I-F fragment of CpGV-M1 was re-sequenced using primer walking and an automated DNA sequencer (D. Winstanley and D. O'Reilly, personal communication). The resulting sequence in the region of the additional DNA present in CpGV-M1, differed significantly from that previously reported in a PhD thesis (Chowdhury, 1992). To confirm the insertion site in the *Sal*I-F region of CpGV-M1 and CpGV-$R3^R$, primers were designed based on the corrected sequence, to amplify the flanking regions of the extra DNA in CpGV-M1 and CpGV-$R3^R$. A region of 4332 bp was sequenced which covered the extra DNA and about 1 kbp either side. The same was sequenced in CpGV-$R3^R$ and found to be identical to CpGV-M1. The equivalent regions in CpGV-R1 and CpGV-R3 were sequenced and found to be identical to each other but contained some differences to CpGV-M1 and CpGV-$R3^R$ in the flanking regions. Therefore, the sites of recombination were not crossed. Sequencing further outwards may not determine the sites of recombination as the sequences between CpGV-R3 and CpGV-M1 appear more similar further away from the extra DNA. An alignment of the sequences is shown in Appendix 10.

Analysis of the corrected CpGV-M1 *Sal*I-F sequence was performed as described in section 2.12.. The coding regions were predicted by locating translation start and stop codons and revealed the presence of ten putative ORFs of over 50 amino acids. Database searches using BLASTP identified similar baculovirus ORFs, Figure 6.16.

Figure 6.16
ORFs over 50 amino acids, defined by start and stop codons, within *Sal*I-F of CpGV-M1 and the equivalent region in CpGV-R1/R3. Arrows indicate direction of transcription.



The variable region upstream of the additional DNA in all genotypes starts within the 5' end of the *p74* gene where there are just two base pair substitutions, Appendix 10, Figure 6.16. These would not affect the gene as both the sequences encode the same amino acids. Downstream of the *p74* gene there is an ORF which has no baculovirus homologues on the database which contains a sequence of 76 base pairs which shows 85.5% DNA identity to a region in another part of the genome (*Sal*I-G), Figure 6.17. The *Sal*I-G region was sequenced by Jehle *et al.* (1995) and was found to contain a palindrome of 31 base pairs ending 6 bp upstream from where a transposon was found to have inserted. The region in *Sal*I-F contains an imperfect palindrome of 31 bp with 27/31 nucleotides showing dyad symmetry. This same region in CpGV-R1 and CpGV-R3 has one base pair substitution and one base pair insertion in relation to CpGV-M1, Figure 6.17. The entire 76 bp sequence could be described as an imperfect palindrome, with the centre showing the most dyad symmetry.

Jehle *et al.*, 1995 isolated mutants of CpGV containing transposons after a mixed infection in *C. leucotreta* larvae of CpGV and ClGV. One virus named MCp5 was found to contain a transposon (TCl4.7) that had entered into a TGTACA palindrome 6 bp downstream from the palindrome sequence mentioned above in *Sal*I-G. The equivalent sequence in *Sal*I-F does not have the TGTACA motif downstream although it does contain a TA in the relative position. The CpGV-M1 extra DNA is not for a further 704 bp downstream but many rearrangements and duplications have occurred around this area when compared to CpGV-R1/R3.

The ORF downstream of this repeated imperfect palindrome is present in CpGV-M1 and CpGV-R1/R3, although it is smaller in CpGV-R1/R3. This is due to the presence of a number of direct repeats in CpGV-M1. The resulting amino acid sequence contains a number of repeated amino acids, which correspond to a hydrophilic region of the protein. This region is absent in the CpGV-R1/R3 protein. There is a baculovirus late promoter motif (ATAAG) associated with this ORF in all CpGV genotypes and the 5' end of this ORF is approximately 40 base pairs from the start of the extra DNA. This ORF has one homologue on the database which is ORF 50 of PxGV (Hashimoto *et al.*, 1999). This homologue does not contain the repeated amino acid sequence of CpGV-M1.

Figure 6.17

The 76 bp imperfect palindrome sequence from CpGV-M1 *Sal*I-G, CpGV-M1 *Sal*I-F and the equivalent sequence in CpGV-R1/R3.
Bold type denotes the bases displaying dyad symmetry

CpGV-M1 *Sal*I-G

ACGAGTCTGGCTTTATTCAA**GAAATTTTAGCGAAAAA**CTT**TTTTTCGCTAAAATCTCGGCGCAAAGCCAGATTCGT**

CpGV-M1 *Sal*I-F

ACGAGTCTGCTTTT**T**TTC**C**A**GAAATTTTAGC**T**A**AAAA**CTT**TTTC**T**CG**GC**AAAA**T**CT**T**GGCGCAAA**A**CCAGA**C**TCGT**

CpGV-R1/R3 equivalent *Sal*I-F sequence

ACGAGTCTGCTTTT**T**TTC**C**A**GAAATTTTAGC**T**A**AAAA**CTT**TTTC**T**CG**GC**AAAA**T**CTCGGCGCAAA**AA**CCAGA**C**TCGT**

= Nucleotides differing from the CpGV-M1 *Sal*I-G sequence

= Central bases of palindrome

There are four potential ORFs of over 50 amino acids within the extra DNA of CpGV in *Sal*I-F, Figure 6.16. The first is a small ORF of 55 amino acids which does not have any similarities to proteins on the database. The second is a homologue of XcGV ORF 66 (Hayakawa *et al.*, 1999). The function of this gene has not yet been determined. There is then another ORF, which is a homologue of AcMNPV ORF 79 and XcGV ORF 75; the functions of these genes have also not been determined. Downstream is a putative protein tyrosine phosphatase (*ptp*) gene with homology to several other *ptps* in baculoviruses and other organisms. Downstream of the extra DNA the sequence from all genotypes appears to be virtually identical with only a few bp changes. Approximately 100 bp downstream of the extra DNA there is a small ORF of 87 amino acids which does not have any similarities to proteins on the database. The ORFs downstream of this are homologues of *p47* and *orf38* of AcMNPV (XcGV *orf79*), Figure 6.16.

6.3 Discussion

All of the four CpGV genotypes were found to be highly pathogenic to neonate larvae, with LD_{80} doses of approximately 10 OBs. The time-mortality (ST_{50}) bioassays showed that there was no statistically significant difference between CpGV-R1, CpGV-R3 and CpGV-$R3^R$. However, there was a statistically significant difference between CpGV-M1 and the Russian genotypes. This result therefore confirms that the 2.45 kbp region is not responsible for the increased speed of kill of CpGV-M1 as compared with CpGV-R1. This means that other differences in the genome must be involved in the speed of kill and these will need investigating.

The bioassays also showed that even though CpGV-M1 consistently killed significantly faster than CpGV-R1, it was not as pronounced as previously reported (Chowdhury, 1992). The difference ranged from 3.5-5.3% faster than CpGV-R1, or on average 6 hours faster. However, the bioassay performed may not have been optimal to see the difference between the viruses. The LD_{80} dose administered may not have been the optimal dose to differentiate between the speeds of kill. An LD_{50} dose or lower may have shown more of a difference, as may have a very high dose (higher than LD_{100}). In understanding how speed of kill is determined for bioinsecticide development, the neonate stage of *C. pomonella* is the most important stage as this is the target instar for control. However, larvae infected as later instars may show a greater difference in speed of kill. More time would have enabled these further investigations to be carried out.

In the regions compared, the genotypes CpGV-R3 and CpGV-R1 have only single base pair differences. A similar level of difference may extend across some or all of the remainder of the genome, or may be restricted only to the characterised area, but these differences could have been enough to affect the properties of the virus by disrupting a gene or promoter of a gene. However, the ST_{50} and LD_{50} of CpGV-R3 and CpGV-R1 seem to be the same *in vivo*. CpGV-R3 probably represented a very small proportion of the CpGV-R1 stock, but it was this genotype that must have recombined with *Sal*I-F to form CpGV-$R3^R$. However, CpGV-R3 could not easily be recovered from cells infected with CpGV-R1.

Another interesting fact to note is that after three or four passages of the recombinant virus, OBs were no longer produced. The cells were infected but OBs were not observed and the resulting virus was not infectious *per os*. A similar scenario has been reported for AcMNPV. After serial passage in cell culture, a spontaneous genomic insertion occurred usually within the *fp25* gene and this resulted in few polyhedra being produced (Beames and Summers, 1989; Fraser *et al.*, 1983; Jarvis *et al.*, 1992). Also a mutant of AcMNPV was detected after serial passages in cell culture using a *Trichoplusia ni* cell-line (Miller and Miller, 1982). It was observed that there appeared to be gradually fewer OBs after each passage of the virus. The virus was purified and it was found that a fragment of DNA had inserted into the *Hin*dIII-K fragment of AcMNPV. This insert was found to be a member of the gypsy family of retrotransposons and was named Transposable Element D (TED). It was a 7.5-kb Ty*3*-like retroelement, which exhibited transcription and expression activities even after insertion into the viral genome (Miller and Miller, 1982). It is possible that an insertion from part of the *Sal*I-F plasmid into the genome may have resulted in the FP-like phenotype in this experiment.

The variable DNA sequence upstream of the insertion in all CpGV genotypes contained an imperfect palindrome within a 76 bp sequence which was also found elsewhere in the genome. It is possible that this may act as a homologous region (*hr*). These *hrs* have been found in all baculoviruses sequenced to date. They comprise direct repeats and imperfect palindromic sequences and have closely related sequences elsewhere in the genome. AcMNPV *hrs* have between one and nine 30 bp imperfect palindromes within a directly repeated sequence (Ayres *et al.*, 1994). Such symmetric sequences have been found to act as origins of replication and as promoters or enhancers of early genes in NPVs where they may have a regulatory function (Krappa *et al.*, 1992; Theilman and Stewart 1992).

The repeats in CpGV appear to have only one palindrome sequence within a 76 bp sequence. However, the absence of any other possible *hrs* from the CpGV sequence to date enforces the idea that these regions may act as *hrs* regions. A *Dpn*1 replication assays will determine this (Leisy and Rohrmann, 1993).

The *Sal*I-F plasmid may contain an origin of replication. If it does then it is possible that the plasmid may replicate in the presence of a CpGV infection. There was hybridisation of several submolar bands to *Bgl*II-G and the vector pBSK+ during selection of the recombinant, which appeared to be a subpopulation of plasmid DNA along with the virus DNA. Some bands appeared faint on the gels but others hybridised strongly. The submolar bands may be the *Sal*I-F plasmid DNA that has replicated or derivatives therefrom. *Hr*-containing plasmid DNA that has transiently replicated in infected cells has been found to be present as concatemers containing various numbers of copies of the plasmid, the DNA being observed as a ladder effect (Leisy and Rohrmann, 1993; Xie *et al.*, 1995). This may explain the abundance of unexpected size fragments containing the *Sal*I-F region of DNA. However, the sizes of these bands of DNA do not represent exact multiples of the original plasmid; many recombination events would have to have occurred to form different sized plasmids. To determine the presence of such plasmids, they would need to be separated undigested on an agarose gel and probed for using vector as well as insert DNA probes.

The additional DNA that hybridised to *Bgl*II-G and pBSK+ was still present in isolates obtained after feeding infected cells to larvae. Plasmid DNA would not be able to infect a larva *per os*, even if the plasmid DNA became co-occluded with virus, as it would not be able to enter the microvilli of midgut cells unless it had been packaged within the virions as well; which is unlikely. Another way this DNA could be present in an infected larva would be if the plasmid had recombined with CpGV. The number of bands that hybridised to the *Bgl*II-G and pBSK+ probes suggests multiple recombination. The differences in intensity of the bands suggests either a population of different recombinant viruses or a virus in which different lengths of the *Sal*I-F plasmid had inserted at several locations. These viruses were likely to be non-occluded, as the passage 4 infected cells contained hardly any OBs but were still highly infected judging by their appearance and the extent of hybridisation to the *Bgl*II-G probe. During virus isolation, the cells infected with the P2 stock virus were frozen at –20°C before feeding to larvae. This temperature eliminates non-occluded and budded virus and therefore only occluded virus would be expected to survive. Therefore, the non-occluded virions may have become co-occluded with the occluded

virions to survive this treatment. The amount of occluded virus appeared to decrease dramatically as the virus was passaged. This may be due to an increase in budded virus formation by the non-occluded virus, which out-competed the budded virus formation produced by the occluded recombinant.

The recombination of *Sal*I-F with CpGV could have formed defective interfering particles (DI). These particles are encoded by genetically deleted virus genomes which lack one or more essential functions for replication (Cann, 1995). The plasmid may have recombined with CpGV to form a background amount of DI particles, which could replicate only when the CpGV genome replicated. The DI particles may have become occluded and hence have been infectious *per os*, although the total lack of OBs within the cells suggests that this may not have been the case.

The larvae infected with this virus looked very different to normal infected larvae, appearing translucent and flaccid and they produced very few OBs. They also took a very long time to die (11-16 days). When these OBs were fed to further larvae, some of the resulting infections were normal, which suggested that a small amount of normal virus was present in the initial OBs, along with the abnormal infection.

The abundance of *Bgl*II-G and pBSK+ homologous fragments in the uncloned recombinant virus isolates suggests that the *Sal*I-F plasmid does contain an *hr* region which allows it to replicate in the presence of CpGV, thereby increasing the chance of recombination events. The correct recombinant was eventually recovered from the mixture when it was passed through cells again and fed to larvae. Once in larvae the virus was cloned by limiting dilution and the virus recovered from some of the infected larvae consisted of only the recombinant occlusion-positive virus.

Major rearrangements, insertions and deletions relative to AcMNPV were detected near all OpMNPV *hrs* (Possee and Rohrmann, 1997). Therefore, it appears that these regions may be 'hot spots' for rearrangements. This could be why the TC14.7 transposon inserted close to the 76 bp region in *Sal*I-G (Jehle *et al.*, 1995) and why there are many differences between CpGV-M1 and CpGV-R1 around this repeated sequence in *Sal*I-F.

The absence of the three baculovirus homologues within the extra region of DNA in CpGV-M1 appears not to be responsible for the decreased virulence of CpGV-R1 and CpGV-R3. CpGV-$R3^R$ did not kill more quickly with these genes present. Therefore, the advantage of these genes as far as virulence and infectivity are concerned is unknown. It is interesting, however, that other GVs and NPVs have maintained these genes throughout evolution.

One of the genes absent for CpGV-R1 encodes a putative protein tyrosine phosphatase (PTP). PTPs are a diverse group of proteins that can be divided into two subfamilies, the receptor and the non-receptor PTPs, depending on whether they span the cell membrane. Based on the lack of any apparent transmembrane motif, the CpGV PTP protein appears to be a member of the non-receptor PTPs. There is a sequence called the HC motif that all protein phosphatases possess, which is (I/V)HCXAGXXR(S/T)G (Charbonneau and Tonks, 1992). The cysteine residue has been shown to be essential for activity (Charbonneau and Tonks, 1992). CpGV PTP does contain this motif although there is no terminal G residue. However, the G residue is also absent from other baculovirus PTP homologues. There appear to be three subfamilies of dual-specificity phosphatases. AcMNPV PTP is within one group along with OpMNPV PTP-1. MbNPV PTP, OpMNPV PTP-2 and CpGV PTP belong to a different group (O'Reilly, 1997). As OpMNPV contains two *ptp* genes, it is possible that other baculoviruses may contain a homologue of each subfamily. It is interesting to note that a homologue of *ptp* was not found in the genome of XcGV (Hayakawa *et al.*, 1999).

There has been some work performed on the *ptp* gene of AcMNPV (Li and Miller, 1995). An AcMNPV recombinant virus was produced which lacked the *ptp* gene (vPTPdel) (Li and Miller, 1995). The resulting recombinant was indistinguishable from the wild-type in *Spodoptera frugiperda* larvae in relation to speed of kill, LD_{50} and yield of OBs (Li and Miller, 1995). However, a phenotype was observed in a *Spodoptera frugiperda* cell line. There appeared to be greater variance in the number of OBs produced per infected cell (Li and Miller, 1995). Some cells produced many OBs whereas others produced very few or none. It was also observed that the budded virus production was less in the recombinant than in the wild-type (Li and Miller, 1995). It is difficult to count the number of granulovirus OBs in infected cells due to

their small size, therefore it is not known whether a similar variation in OB production occurred in CpGV-R1 or CpGV-R3 infected cells as compared to CpGV-M1. In addition, CpGV-R1/R3 budded virus production did not appear to be any less than that of CpGV-M1.

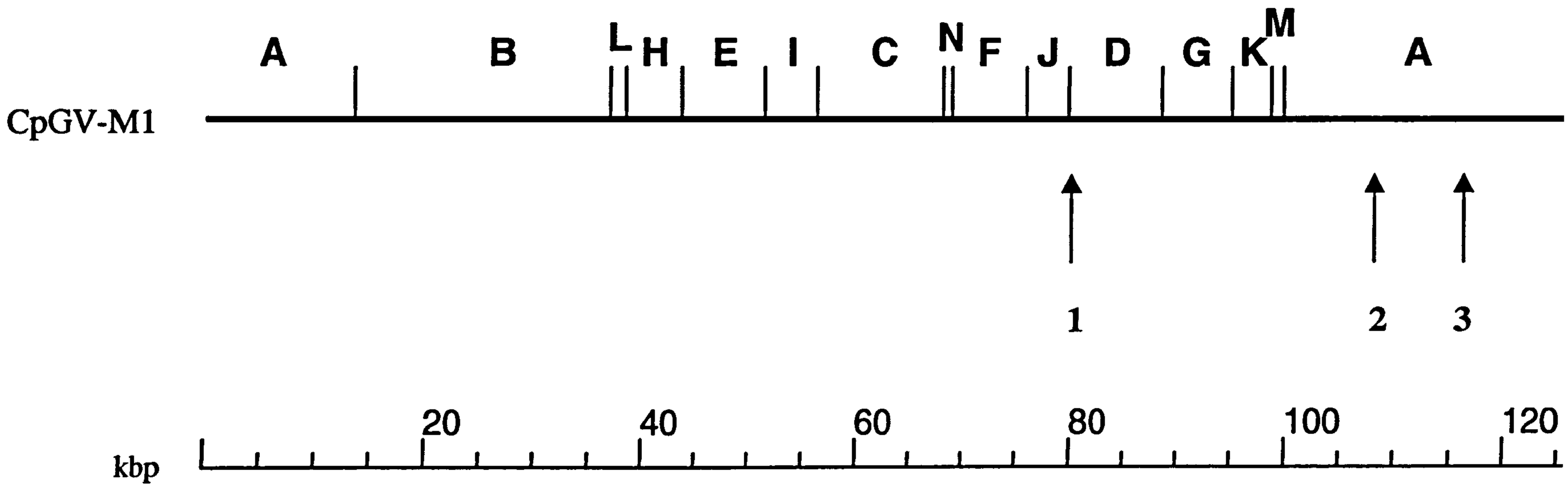
The 1 kbp region of DNA upstream of the inserted region, between it and the 5' end of the *p74* gene in CpGV-R3^R is the same as CpGV-M1 and not CpGV-R1. Therefore, recombination must have occurred within the *p74* gene of *Sal*I-F. Only the first 32 bp of this gene has been sequenced in CpGV-R1 and it contained two base pair substitutions in relation to CpGV-M1. The CpGV-R1 and CpGV-M1 DNA appear to have greater similarity further away from extra DNA, therefore the differences within this gene are likely to be very small. Therefore, the exact site of recombination may not be detectable. The fact that recombination occurred within the *p74* gene means that the imperfect palindrome and the ORF containing the direct repeats are also not involved in the increased speed of kill of CpGV-M1. This is because CpGV-R3^R contains the CpGV-M1 copies of these genes. Therefore, the area of the genome responsible for this increase in speed of kill is elsewhere.

There are further restriction site differences between CpGV-M1 and CpGV-R1, Figure 6.18. The extra *Xho*I site in CpGV-M1 is within an ORF. This site is within a homologue of ORF 93 of AcMNPV, which has an unknown function (Ayres *et al.*, 1994). Other restriction site differences may become apparent using further restriction enzymes. These areas should be the first to investigate. However, it is possible there could be base pair substitutions spread along the whole of the genome. These are unlikely to be detected using restriction profiles but could be enough to alter the speed of kill of the virus. Only complete sequencing would reveal the full extent of differences between these two isolates of CpGV.

The reason for the extra 2.45 kbp in CpGV-M1 was originally thought to be due to transposition from the host *C. pomonella* DNA (Chowdhury, 1992). However, the extra DNA does not have any transposon-like features and the fact that the DNA contains three baculovirus homologues suggests that this is more likely to be a viral sequence that had been lost from CpGV-R1. There is a region of about 1 kbp in CpGV-M1 that contains only one small potential ORF of 55 amino acids. This is

Figure 6.18

CpGV-M1 *Xho*I restriction map. Arrows indicate restriction site differences between CpGV-M1 and CpGV-R1/R3 that are not associated with the 2.45 kbp extra DNA.



1 = There is an extra *Xho*I site in CpGV-M1

2+3= There are 2 extra *Pst*I site in CpGV-R1/R3. The precise location is not known, but they are within CpGV-M1 *Pst*I-A and *Xho*I-A.

unusual for baculovirus genomes, which are usually densely packed with genes with the exception of *hr* regions. This 1 kbp region did not appear to contain any *hr*-like regions although there was a small region present that contained some short dyads and direct repeats. The DNA within this region may have been acquired from the host or another organism. Hybridisation of the *Sal*I-F region of CpGV-M1 to *C. pomonella* genomic DNA did not result in any detectable hybridising fragments (data not shown). It is possible that due to the putative *hr* region (palindrome) upstream, a deletion of the three baculovirus homologue ORFs occurred in the CpGV-M to form CpGV-R and subsequently an insertion of some extraneous DNA occurred in the CpGV-M genome. However, no effect on the infectivity or speed of kill of the viruses has occurred due to these changes.

CHAPTER 7

Summary and Future Work

The primary aim of this project was to expand the information known on the speed of kill, host range and pathology of granuloviruses. Initial studies focused on the genomic relationship of GVs involving DNA hybridisations between fast and slow GVs. From the results it appeared that GVs that infect lepidopteran larvae from the same family were more closely related in terms of genomic similarity and genome organisation. Prior to these studies GVs had been categorised into two groups, fast killing and slow killing (Federici, 1997). The hybridisation studies were performed to see how closely related fast GVs were to each other and to slow GVs. It was during these studies that it became apparent that the fast GV CpGV was similar and collinear to the slow GV AoGV and both these GVs infect tortricid larvae. The slow GVs XcGV and TnGV, which both infect noctuid insects were found to be very similar. An exception was LoGV, which infects noctuids, but its genome appeared relatively similar to CpGV. Further studies should be undertaken to compare LoGV with other noctuid infecting virus e.g. TnGV. LoGV is known to contain an *egt* gene and its genome is more similar in size to GVs that do not infect noctuids. It is also known to be fast killing. Therefore, this virus could represent an intermediate between the slow noctuid infecting GVs and fast GVs that infect other families. PxGV is the only GV used in these studies that infects larvae of the family *Yponomeutidae*. In all the phylogenetic analyses this virus grouped with the slow noctuid infecting GVs rather than the fast GVs that infect other families. The slow GV AoGV appeared to be very interesting. It contained a relatively small genome of approximately 100.9 kbp, as determined by the mapping. This was over 20 kbp less than CpGV and is one of the smallest GVs known. This suggests that a higher proportion of the genetic information packed into this genome may be essential and evolutionarily fundamental for baculoviruses compared to larger baculovirus genomes. The sequencing of the fast GV CpGV genome is now very near completion. Also, the genome of PxGV, another fast GV of similar size and XcGV a slow GV with a large genome have now both been completely sequenced (Hashimoto *et al.*, 1999; Hayakawa *et al.*, 1999). A comparison of the genes shared by all GVs will determine genes essential to GVs. Any additional genes will be interesting to study as these may be involved in things such as host range, tissue tropism and speed of kill. The fact that a small slow GV genome (AoGV) can be compared to small fast GV genomes (CpGV and PxGV) will be useful in the search for genes or factors involved in host range, tissue tropism and speed of kill. This work has initiated a project to completely sequence the AoGV

genome, in which I will be directly involved. Certain areas of the AoGV genome have been targeted to study first. These are the areas revealed by the hybridisation studies that do not hybridise to CpGV.

A region of 4460 bp of AoGV was sequenced during these studies so that the organisation of genes around the granulin area could be compared to other GVs and phylogenetic analysis could be performed on genes common to GVs. These studies supported the hypothesis that the relatedness of GVs appears to be determined by the family of the larvae they infect rather than their speed of kill.

The results in Chapter 4 presented an initial investigation into the slow GV AoGV. Bioassays were performed to determine its infectivity and speed of kill, the genome was mapped to enable hybridisation studies with other GVs and as mentioned above the granulin-containing region of the genome was sequenced for comparison to other GV homologues. The LD_{50} values of AoGV in *A. orana* larvae increased dramatically as the instar in which they were infected increased. The degree of virus resistance in older instars appears to be determined by the specific virus/host association. Some infections such as CpGV in *C. pomonella* neonates require just one OB for a median lethal dose and have an LD_{50} of only ten OBs for fifth instar larvae (Chowdhury, 1992). Other infections require large amounts of OBs in all instars. In the latter cases these hosts may not be the native host and the virus may be more infectious to an alternative host. However, *C. leucotreta* which is less susceptible to CpGV than *C. pomonella* still requires relatively few OBs (around 30) to cause infection in neonates but a huge amount in fifth instar (around 7×10^6) (D. Winstanley, personal communication). These latter figures are similar to those of AoGV in *A. orana* larvae, which may suggest that a more permissive host may exist or may have existed. AoGV has also been found to infect the closely related species the smaller tea tortrix. However, this host does not appear to be any more permissive from preliminary studies undertaken (Y. Kunimi, personal communication). Future work should be undertaken to compare the biology of AoGV in these two hosts. One interesting observation during AoGV infection in *A. orana* is that the majority of larvae die as larvae/pupae intermediates, which suggests some hormonal disruption during the late stages of infection. This is much less common in AoGV infected smaller tea tortrix larvae and occurs usually only when a high dose is administered,

therefore a study of enzyme levels such as JHE and EGT in the infections of these two hosts would be very interesting to perform.

The median survival time (ST_{50}) bioassays showed that regardless of which instar the larvae are infected, they do not die until in the final instar. This is also the case for other slow killing viruses such as TnGV and XcGV although AoGV appears to be much slower to kill than these other slow killing viruses. The lack of an *egt* gene seemed an obvious explanation for the ability to continue moulting. However, AoGV was found to contain an *egt* homologue and produce a functional EGT able of the conjugating UDP-sugars and ecdysone in the final instar. It is possible that the *egt* gene may not become active until very late in infection. The larvae when infected with an LD_{80} dose of AoGV do not appear to behave any differently to control larvae until fifth instar and therefore it seems that the infection does not progress dramatically until fifth instar is reached. Therefore, a threshold level of EGT may not be achieved until the fifth instar. The fifth instar is prolonged for about 20 days longer than usual. The larvae partially pupate late in infection perhaps due to a drop of EGT or disruption of JHE levels. Another possibility for the continuation of larval moulting is that due to the narrow tissue tropism of AoGV, there is not enough EGT produced to reach the required threshold level to inhibit moulting until late in infection.

The virus exudate emitted late in infection is also atypical of GV infections. The only other GV known to cause viral discharge is *Harrisina brillians* GV (HbGV) in *H. brillians*. This GV infects only the midgut, which results in chronic diarrhoea. The EM studies indicated a possible midgut infection although more work will have to be done to confirm this. Nevertheless, the discharge of virus is an interesting strategy to disseminate the virus throughout infection in multiple locations, rather than only once the larvae die in one location as in most baculovirus infections. This has implications for its use as a biocontrol agent in relation to prolonged control. The slow kill of the virus results in control within the same generation and overlapping generations, due to the discharge of virus. It is also possible for AoGV infected *A. orana* larvae to overwinter as second or third instar larvae and reintroduce the virus the following season.

The EM studies showed that by 4.5 d p.i. larvae infected as fourth instar, which did not show any external signs of infection, actually contained some highly infected fat body cells. These larvae were in fifth instar by the time they were dissected. It would be very interesting to repeat the EM studies with neonates infected with AoGV and follow them through the instars to see exactly when and where budded virions and OBs can be observed.

The original virus collected from the field was a mix of GV and NPV, the NPV will need to be completely separated from the GV before further studies are performed. The presence of large aggregated masses of GVs within a matrix, may be responsible for the unsuccessful separation of NPV from GV using gradients. Mixes of NPV and GV may actually result in a more effective biocontrol agent as there may be some synergism between the two. If AoGV encodes an enhancin gene then the entry of NPV through the peritrophic membrane will be enhanced and it will be an advantage for the NPV to coinfect with the GV. Studies to investigate the efficacy of mixes of NPV and GV will be performed once the NPV has been purified.

The work discussed in Chapter 5 focuses on the production of recombinant GVs with either an expanded host range or insertion of a *lacZ* gene into the genome. The GV system for producing recombinants is still in its infancy. The cell line used has been found to be very fastidious in its requirements and the m.o.i. achievable is a fraction of that achievable in NPV systems. Plaque assays are not feasible due to the very low percentage agar required for the sensitive *C. pomonella* cells. Therefore selection of recombinant viruses using the dilution method is often laborious and time consuming. However, recombination does occur within these cells and a *lacZ* positive recombinant of CpGV-EGT⁻ was selected by staining the cells with X-gal. This appears a much more sensitive approach of detecting recombinants than dot blotting. The *lacZ* positive virus is to be purified and used to study the course of infection of CpGV in various hosts of differing permissivity. This will provide a great insight into the infection process of GVs and the barriers to infection in semi-permissive and non-permissive hosts as has been observed in NPVs in the studies of Washburn *et al.* (1996), Engelhard *et al.* (1994) and Morris and Miller, (1992).

The attempt to expand the host range of ClGV to include *C. pomonella* cells resulted in a promising outcome of which more work will be required. A well containing ClGV DNA was detected and so it appears that CpGV can complement and allow replication of ClGV in *C. pomonella* cells. No progeny virus that was infectious to cells or larvae could be recovered. No OBs were observed in the selected wells where ClGV replication had occurred which would indicate an abortive infection. However, selection of further wells where ClGV replication has been rescued may result in the cloning of a recombinant ClGV that can replicate in *C. pomonella* cells. These studies may lead to the discovery of some of the mechanisms determining host range in GVs.

The final chapter in this thesis involved the various genotypes of the fast GV CpGV. CpGV-R1 was found to be slower killing than CpGV-M1 and preliminary studies of the genomes identified a 2.45 kbp deletion in CpGV-R1 compared with CpGV-M1. To test whether the deletion was directly involved in speed of kill it was inserted into the genome of CpGV-R1 by homologous recombination. A recombinant was detected by dot blotting but found to be a closely related genotype to CpGV-R1 named CpGV-R3 which was also slow to kill. The 2.45 kbp region of DNA was unable to restore the speed of kill of CpGV-R3 and so it was concluded that this region did not contain factors directly responsible for speed of kill but that other differences in the genome must be responsible. There are other regions where restriction sites are absent compared to CpGV-M1 and these will be the first places to investigate. However, there may be numerous regions that differ by small amounts along the genome that are not detectable by restriction digest analysis.

These studies determined a putative origin of replication in the CpGV-M1 genome. The *Sal*I-F sequence contains a palindrome, which is repeated at least once elsewhere in the genome. The *Sal*I-F plasmid DNA appeared to be able to replicate in the *C. pomonella* cells in the presence of CpGV and resulted in a non-occluded virus probably by recombining with the CpGV DNA and resulting in the loss of OB production. The plasmid DNA was also present in larvae that were fed infected cells. This suggested that multiple recombination events had occurred in which pBSK+ had also inserted into the genome. This kind of plasmid 'activity' has not been observed before in *C. pomonella* cells and it may be that the plasmid replication within the cells

resulted in higher recombination efficiency. The palindrome will be tested in a *Dpn*I replication assay for its ability to replicate in the presence of CpGV.

A summary of future work is shown below:

- Sequence the regions of AoGV that did not hybridise to CpGV or TnGV leading to the sequencing of the whole genome.
- Purify AoNPV-E from AoGV-E, perform time and dose mortality bioassays of AoNPV in *A. orana* and smaller tea tortrix. Investigate mixes of the two.
- Perform juvenile hormone esterase assays on AoGV infected *A. orana* and tea tree tortrix larvae.
- Repeat EGT assay using different instars of *A. orana* larvae infected as neonates.
- Perform an extensive electron microscopy study to investigate the tissues *of A. orana* that support AoGV replication and to study the course of infection using larvae infected as neonates.
- Perform a transient expression assay using AoGV EGT to confirm that the AoGV *egt* sequence codes for the functional EGT.
- Clone the recombinant virus CpGV EGT$^-$-*hsp-lacZ* and select the recombinant virus CpGV EGT$^-$-*gran-lacZ*. Use these recombinant GVs to study the course of infection in various hosts.
- Repeat the study to identify host range determinants using ClGV and restricted CpGV DNA to construct a ClGV virus capable of replicating in *C. pomonella* cells.
- Investigate other differences between CpGV-R1/R3 and CpGV-M1 that may be responsible for the increased speed of kill of CpGV-M1.

Most research raises more questions than it answers and the work presented in this thesis is no exception. .However, sufficient progress has been made in this first attempt to compare the slow and fast killing GVs to be able to identify specific questions and to prioritise the direction of the research.

References

Ahrens, C. H., Russell, R. L. Q., Kunk, C. J., Evans, J. T., Harwood, S. H. and Rohrmann, G. F. (1997). The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* **229**, 381-399.

Andermatt, M. (1989). Field efficacy and environmental persistance of the granulosis viruses of the summer fruit tortrix, *Adoxophyes orana* F. v. R. In *Microbial control of pests*, pp. 73-74. Edited by H. F. Evans. Rome.

Argaud, O., Croizier, L., Lopez-Ferber, M. and Croizier, G. (1998). Two key mutations in the host-range specificity domain of the p143 gene of *Autographa californica* nucleopolyhedrovirus are required to kill *Bombyx mori* larvae. *Journal of General Virology* **79**, 931-935.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M. and Possee, R. D. (1994). The complete DNA-sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586-605.

Bah, A., Lucarotti, C. J., Arella, M. and Guertin, C. (1999). *Choristoneura fumiferana* granulovirus: sequence analysis and 5' characterization of ORF891. *Archives of Virology* **144**, 737-746.

Baylis, S. A., Banham, A. H., Vydelingum, S., Dixon, L. K. and Smith, G. L. (1993). African swine fever virus encodes a serine protein-kinase which is packaged into virions. *Journal of Virology* **67**, 4549-4556.

Beames, B. and Summers, M. D. (1989). Location and nucleotide-sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. *Virology* **168**, 344-353.

Beniya, H., Funk, C. J., Rohrmann, G. F. and Weaver, R. F. (1996). Purification of a virus-induced RNA polymerase from *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells that accurately initiates late and very late transcription *in vitro*. *Virology* **216**, 12-19.

Birnbaum, M. J., Clem, R. J. and Miller, L. K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with cys/his sequence motif. *Journal of Virology* **68**, 2521-2528.

Bischoff, D. S. and Slavicek, J. M. (1994). Identification and characterization of a protein kinase gene in the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. *Journal of Virology* **68**, 1728-1736.

Black, B. C., Brennan, L. A., Dierks, P. M. and Gard, I. E. (1997). Commercialization of baculoviral insecticides. In *The Baculoviruses*, pp. 341-387. Edited by L. K. Miller. New York: Plenum Press.

Blissard, G. W. and Rohrmann, G. F. (1990). Baculovirus diversity and molecular-biology. *Annual Review of Entomology* **35**, 127-155.

Bonning, B. C. and Hammock, B. D. (1996). Development of recombinant baculoviruses for insect control. *Annual Review of Entomology* **41**, 191-210.

Bonning, B. C., Hoover, K., Booth, T. F., Duffey, S. and Hammock, B. D. (1995). Development of a recombinant baculovirus expressing a modified juvenile-hormone esterase with potential for insect control. *Archives of Insect Biochemistry and Physiology* **30**, 177-194.

Bonning, B. C., Possee, R. D. and Hammock, B. D. (1999). Insecticidal efficacy of a recombinant baculovirus expressing JHE-KK a modified Juvenile hormone esterase. *Journal of Invertebrate Pathology* **73**, 234-236.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.

Charbonneau, H. and Tonks, N. K. (1992). 1002 protein phosphatases. *Annual Review of Cell Biology* **8**, 463-493.

Charlton, C. A. and Volkman, L. E. (1993). Penetration of *Autographa californica* nuclear polyhedrosis virus nucleocapsids into IPLB SF-21 cells induces actin cable formation. *Virology* **197**, 245-254.

Chen, C. J., Quentin, M. E., Brennan, L. A., Kukel, C. and Thiem, S. M. (1998). Lymantria dispar nucleopolyhedrovirus *hrf-1* expands the larval host range of *Autographa californica* nucleopolyhedrovirus. *Journal of Virology* **72**, 2526-2531.

Chowdhury, S. (1992). Characterization of some aspects of the isolates of *Cydia pomonella* granulosis virus (CpGV). In Department of Microbiology. Reading: Reading University.

Clarke, E. E., Tristem, M., Cory, J. S. and Oreilly, D. R. (1996). Characterization of the ecdysteroid UDP-glucosyltransferase gene from *Mamestra brassicae* nucleopolyhedrovirus. *Journal of General Virology* **77**, 2865-2871.

Collyer, E. (1953). Biology of some predatory insects and mites associated with fruit tree red spider mite IV. The predator-mite relationship. *J. Gen. Virol.* **66**, 2649-2658.

Cory, J. S. and Bishop, D. H. L. (1997). Use of baculoviruses as biological insecticides. *Molecular Biotechnology* **7**, 303-313.

Croizier, G., Croizier, L., Argaud, O. and Poudevigne, D. (1994). Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the *p143* helicase gene. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 48-52.

Crook, N. E. (1991). Baculoviridae: subgroup B. Comparitive aspects of granulosis viruses. In *Viruses of Invertebrates*, pp. 73-110. Edited by E. Kurstak. New York: Dekker, M.

Crook, N. E., Spencer, R. A., Payne, C. C. and Leisy, D. J. (1985). Variation in *Cydia pomonella* granulosis virus isolates and physical maps of the DNA from 3 variants. *Journal of General Virology* **66**, 2423-2430.

Crook, N. E., Clem, R. J. and Miller, L. K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif, *Journal of virology* **67**, 2168-2174.

Crook, N. E., James, J. D., Smith, I. R. L. and Winstanley, D. (1997). Comprehensive physical map of the *Cydia pomonella* granulovirus genome and sequence analysis of the granulin gene region. *Journal of General Virology* **78**, 965-974.

Cross, J. (1994). Apple enemy number one. *Grower* **121**, 18-19.

Cross, J. (1997). Insegar intervention. *Grower* **127**, 15-16.

Cross, J. V., Solomon, M. G., Chandler, D., Jarrett, P., Richardson, P. N., Winstanley, D., Bathon, H., Huber, J., Keller, B., Langenbruch, G. A. and Zimmerman, G. (1999). Biocontrol of pests of apples and pears in northern and central Europe: 1. Microbial agents and nematodes. *Biocontrol Science and Technology* **9**, 125-149.

Darbon, H., Zlotkin, E., Kopeyan, C., Vanrietschoten, J. and Rochat, H. (1982). Covalent structure of the insect toxin of the North African scorpion *Androctonus australis* hector. *International Journal of Peptide and Protein Research* **20**, 320-330.

Derksen, A. C. G. and Granados, R. R. (1988). Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. *Virology* **167**, 242-250.

Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic acids Research* **12**, 387-395.

Drolet, J. (1989). The nuclear and the cytoplasmic granulosis viruses of *Adoxophyes orana* F.V.R. (Lep., Tortricidae) : Separation, biochemical characterization, pathogenesis and infectivity. Swiss Federal Institute of Technology: Zurich.

Dwyer, K. G, Granados, R. R. (1987). A physical map of the *Pieris rapae* granulosis virus genome. *Journal of General Virology* **68**, 1471-1476.

Eldridge, R., O'Reilly, D. R. and Miller, L. K. (1992). Efficacy of a baculovirus insecticide expressing an eclosion hormone gene. *Biol. Control* **2**, 104-110.

Engelhard, E. K., Kammorgan, L. N. W., Washburn, J. O. and Volkman, L. E. (1994). The insect tracheal system - a conduit for the systemic spread of *Autographa californica* m nuclear polyhedrosis virus. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 3224-3227.

Evans, O. P. and O'Reilly, D. R. (1998). Purification and kinetic analysis of a baculovirus ecdysteroid UDP-glucosyltransferase. *Biochemical Journal* **330**, 1265-1270.

Falcon, L. A., Kane, W. R. and Bethell, R. S. (1968). Preliminary evaluation of a granulosis virus for control of codling moth. *J. Econ. Entomol.* **61**, 1208-1213.

Faulkner, P. (1981), Baculovirus. In: *Pathogenesis of invertebrate microbial diseases*, pp. 3-33, Edited by E. W. Davidson. Allanheld, Osmun, Totowa, NJ.

Federici, B. A. (1997). Baculovirus Pathogenesis. In *The Baculoviruses*, pp. 33-59. Edited by L. K. Miller. New York: Plenum Press.

Finley, D. and Chau, V. (1991). Ubiquitination. *Annual Review of Cell Biology* **7**, 25-69.

Finney, D. J. (1971). Probit analysis, 3rd edn. Cambridge, England: Cambridge University Press.

Flipsen, J. T. M., Mans, R. M. W., Kleefsman, A. W. F., Knebel-Mörsdorf, D. and Vlak, J. M. (1995). Deletion of the baculovirus ecdysteroid UDP-glucosyltransferase gene induces early degeneration of malpighian tubules in infected insects. *Journal of Virology* **69**, 7380-7381.

Fraser, M. J., Smith, G. E. and Summers, M. D. (1983). Acquisition of host-cell DNA sequences by baculoviruses - relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. *Journal of Virology* **47**, 287-300.

Fraser, M. J., Cary, L., Boonvisudi, K., and Wang, H. G. H. (1995). Assay for movement of lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA, *Virology* **211**, 397-407.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B. and Yamamoto, K. R. (1988). The function and structure of the metal coordination sites within the glucocorticoid receptor DNA-binding domain. *Nature* **334**, 543-546.

Friesen, P. D. (1997). Regulation of baculovirus early gene expression. In *The Baculoviruses*, pp. 141-170. Edited by L. K. Miller. New York: Plenum Press.

Fuchs, L. Y., Woods, M. S. and Weaver, R. F. (1983). Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection - a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *Journal of Virology* **48**, 641-646.

Funk, C. J. and Consigli, R. A. (1993). Phosphate cycling on the basic-protein of *Plodia interpunctella* granulosis virus. *Virology* **193**, 396-402.

Glen, D. M. and Clark, J. (1985). Death of *Cydia pomonella* larvae and damage to apple fruit, after field application of codling moth granulosis virus. *Entomologia Experimentalis et Applicata* **38**, 93-96.

Glen, D. M. and Payne, C. C. (1984). Production and field evaluation of codling moth granulosis virus for control of *Cydia pomonella* in the United Kingdom. *Annals of Applied Biology* **104**, 87-98.

Glen, D. M., Milson, N. F. and Scutt, V. J. (1976). Population ecology of codling moth in a cider orchard, pp. 90-91: Long Ashton Research Station.

Gomi, S., Majima, K. and Maeda, S. (1999). Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *Journal of General Virology* **80**, 1323-1337.

Gonzalez, R. H. (1982). Establishing pest management system in deciduous fruit trees in Chile. In *The role of biological control in pest management*, pp. 21-29. Edited by G. Allen and A. Rada: University of Otawa press.

Gopalakrishnan, B., Muthukrishnan, S. and Kramer, K. J. (1995). Baculovirus-mediated expression of a *Manduca sexta* chitinase gene - properties of the recombinant protein. *Insect Biochemistry and Molecular Biology* **25**, 255-265.

Goto, C., Minobe, Y. and Iizuka, T. (1992). Restriction endonuclease analysis and mapping of the genomes of granulosis viruses isolated from *Xestia c-nigrum* and five other noctuid species. *Journal of General Virology* **73**, 1491-1497.

Granados, R. R. and Lawler, K. A. (1981). *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology* **108**, 297-308.

Granados, R. R., Derksen, A. C. G. and Dwyer, K. G. (1986). Replication of the *Trichoplusia ni* granulosis and nuclear polyhedrosis viruses in cell cultures. *Virology* **152**, 472-476.

Gröner, A. (1986). Specificity and safety of baculoviruses, in *The Biology of Baculoviruses*, vol.1, pp.177. Edited by R. R. Granados and B. A. Federici. Boca Raton, FL: CRC Press.

Grula, M. A., Buller, P. L. and Weaver, R. F. (1981). Alpha-amanitin resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. *Journal of Virology* **38**, 916-921.

Grula, M. A. and Weaver, R. F. (1981). An improved method for isolation of *Heliothis zea* DNA-dependent RNA-polymerase - separation and characterization of a form-III RNA-polymerase activity. *Insect Biochemistry* **11**, 149-154.

Guarino, L. A., Gonzalez, M. A. and Summers, M. D. (1986). Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **60**, 224-229.

APPENDIX 4

APPENDIX TABLE 4.1

Neonate bioassay LD_{50} data

	BIOASSAY 1			BIOASSAY 2			BIOASSAY 3		
Dose (OBs)	No. infected	No. dead	% dead	No. infected	No. dead	% dead	No. infected	No. dead	% dead
5	45	6	13.33	39	4	10.26	42	5	11.90
15	42	11	26.19	44	14	31.82	46	11	23.91
45	42	25	59.52	43	22	51.16	39	30	76.92
135	42	37	88.10	44	35	79.55	42	35	83.33
405	45	44	97.78	46	46	100.0	38	37	97.37

APPENDIX TABLE 4.2

Fourth instar bioassay LD_{50} data

	BIOASSAY 1			BIOASSAY 2			BIOASSAY 3		
Dose (OBs)	No. infected	No. dead	% dead	No. infected	No. dead	% dead	No. infected	No. dead	% dead
$2x10^2$	39	4	10.26	49	4	81.63	45	1	2.22
$1x10^3$	37	11	29.73	47	5	10.64	49	17	34.69
$5x10^3$	43	28	65.12	47	11	23.40	45	19	42.22
$2.5x10^4$	33	27	81.82	46	32	69.57	40	28	70.00
$1.25x10^5$	38	36	94.74	44	35	79.55	41	39	95.12

Guarino, L. A. and Summers, M. D. (1986). The homologous DNA of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) transactivates viral transcription. *Journal of Cellular Biochemistry*, 185.

Guzo, D., Rathburn, H., Guthrie, K. and Dougherty, E. (1992). Viral and host cellular transcription in *Autographa californica* nuclear polyhedrosis virus-infected gypsy moth cell lines. *Journal of Virology* **66**, 2966-2972.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988). The protein kinase family - conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Harvey, J. P. and Volkman, L. E. (1983). Biochemical and biological variation of *Cydia pomonella* (codling moth) granulosis virus. *Virology* **124**, 21-34.

Hashimoto , Y. and Hayakawa, T. (1999). Comparison of the genome organisation of granuloviruses from *Plutella xylostella* and *Xestia c-nigrum*, In *Society of Invertebrate Pathology 32nd meeting, 22-27 August 1999, University of California at Irvine*, pp 43.

Hashimoto, Y., Fujita, T. and Ueno, Y. (1999). DNA sequence analysis *Plutella xylostella* granulovirus. *RIKEN Review* **22**, 30-33.

Hashimoto, Y., Hayashi, K., Hayakawa, T., Ueno, Y., Shimojo, E., Kondo, A., Miyasono, M., Sano, Y., Matsumoto, T. and Granados, R. R. (2000). Physical map of a *Plutella xylostella* granulovirus genome. *Applied Entomology and Zoology* **35**, 45-51.

Hashimoto, Y., Hayashi, K., Okuno, Y., Hayakawa, T., Saimoto, A., Granados, R. R. and Matsumoto, T. (1996). Physical mapping and identification of interspersed homologous sequences in the *Trichoplusia ni* granulosis virus genome. *Journal of General Virology* **77**, 555-563.

Hawtin, R. E., King, L. A. and Possee, R. D. (1992). Prospects for the development of a genetically engineered baculovirus insecticide. *Pesticide Science* **34**, 9-15.

Hawtin, R. E., Arnold, K., Ayres, M. D., Zanotto, P. M. D., Howard, S. C., Gooday, G. W., Chappell, L. H., Kitts, P. A., King, L. A. and Possee, R. D. (1995). Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212**, 673-685.

Hawtin, R. E., Zarkowska, T., Arnold, K., Thomas, C. J., Gooday, G. W., King, L. A., Kuzio, J. A. and Possee, R. D. (1997). Liquefaction of *Autographa californica* nucleopolyhedrovirus infected insects is dependant on the integrity of virus-encoded chitinase and cathepsin genes. *Virology* **238**, 243-253.

Hayakawa, T., Ko, R., Okano, K., Seong, S. I., Goto, C. and Maeda, S. (1999). Sequence analysis of the *Xestia c-nigrum* granulovirus genome. *Virology* **262**, 277-297.

Hayakawa, T., Shimojo, E., Mori, M., Kaido, M., Furusawa, I., Miyata, S., Sano, Y., Matsumoto, T., Hashimoto, Y. and Granados, R. R. (2000). Enhancement of baculovirus infection in *Spodoptera exigua* (Lepidoptera : Noctuidae) larvae with *Autographa californica* nucleopolyhedrovirus or *Nicotiana tabacum* engineered with a granulovirus enhancin gene. *Applied Entomology and Zoology* **35**, 163-170.

Higgins, D. G. and Sharp, P. M. (1988). CLUSTAL V: a package for performing multiple sequence alignment on a micro computer. *Gene* **73**, 237-244.

Hill, D. S. (1987). Agricultural insect pests of temperate regions and their control. Cambridge: Cambridge University Press.

Honma, K. (1970). Morphological difference of the smaller tea tortrix, *Adoxophyes orana*, in Japan. *Jap. J. Appl. Ent. Zool.* **14**, 89-94.

Howley, P. M., Israel, M. A., Law, M.F. and Martin, M. A. (1979). A rapid method for detecting and mapping homology between heterologous DNAs. *Journal of Biological Chemistry* **254**, 4876-4883

Hu, Z. H., Broer, R., Westerlaken, J., Martens, J. W. M., Jin, F., Jehle, J. A., Wang, L. M. and Vlak, J. M. (1997). Characterization of the ecdysteroid UDP-glucosyltransferase gene of a single nucleocapsid nucleopolyhedrovirus *of Buzura suppressaria. Virus Research* **47**, 91-97.

Huber J. (1982). The baculoviruses of *Cydia pomonella* and other tortricids, in *Invertebrate Pathology and Microbial Control, Proceedings Third International Colloquium on Invertebrate Pathology, 6-10 September 1982, Brighton, UK.*, pp.119.

Huber, J. and Dickler, E. (1977). Codling moth granulosis virus: its efficiency in the field in comparison with organophosphorus insecticides. *Journal of Economic Entomology* **70**, 557-561.

Hughes, P. R., van Beek, N. A. M. and Wood, H. A. (1986). A modified droplet feeding method for rapid assay of *Bacillus thuringiensis* and baculoviruses in noctuid larvae. *Journal of Invertebrate Pathology* **48**, 187-192.

Hughes, P. R. and Wood, H. A. (1981). A synchronous peroral technique for the bioassay of insect viruses. *Journal of Invertebrate Pathology* **37**, 154-159.

Hughes, P. R., Wood, H. A., Breen, J. P., Simpson, S. F., Duggan, A. J. and Dybas, J. A. (1997). Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. *Journal of Invertebrate Pathology* **69**, 112-118.

Huh, N. E. and Weaver, R. F. (1990). Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* **71**, 195-201.

Hunter-Fujita, F. R., Entwistle, P. F., Evans, H. F. and Crook, N. E. (1998). Insect Viruses and Pest Management. Chichester: Wiley.

Ijkel, W. F. J., van Strien, E. A., Heldens, J. G. M., Broer, R., Zuidema, D., Goldbach, R. W. and Vlak, J. M. (1999). Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *Journal of General Virology* **80**, 3289-3304.

Jarvis, D. L., Bohlmeyer, D. A. and Garcia, A. (1992). Enhancement of polyhedrin nuclear-localization during baculovirus infection. *Journal of Virology* **66**, 6903-6911.

Jarvis, D. L., Weinkauf, C. and Guarino, L. A. (1996). Immediate-early baculovirus vectors for gene expression in transformed or infected insect cells. *Protein Expression and Purification* **8**, 191-203.

Jehle, J. A. and Backhaus, H. (1994). The granulin gene region of *Cryptophlebia leucotreta* granulosis virus - sequence analysis and phylogenetic considerations. *Journal of General Virology* **75**, 3667-3671.

Jehle, J. A., Backhaus, H., Fritsch, E. and Huber, J. (1992). Physical map of the *Cryptophlebia leucotreta* granulosis virus genome and its relationship to the genome of *Cydia pomonella* granulosis virus. *Journal of General Virology* **73**, 1621-1626.

Jehle, J. A., Fritsch, E., Nickel, A., Huber, J. and Backhaus, H. (1995). TC14.7 - A novel lepidopteran transposon found in *Cydia pomonella* granulosis virus. *Virology* **207**, 369-379.

Joel Funk, C., Braunagel. C. and Rohrmann, G.F. (1997). Baculovirus Structure. In *The Baculoviruses*, pp. 7-32. Edited by L. K. Miller. New York: Plenum Press.

Kamita, S. G. and Maeda, S. (1993). Inhibition of *Bombyx mori* nuclear polyhedrosis virus (NPV) replication by the putative helicase gene of *Autographa californica* NPV. *Journal of Virology* **67**, 6239-6245.

Kamita, S. G. and Maeda, S. (1996). Abortive infection of the *baculovirus Autographa californica* nuclear polyhedrosis virus in Sf-9 cells after mutation of the putative DNA helicase gene. *Journal of Virology* **70**, 6244-6250.

Kamita, S. G. and Maeda, S. (1997). Sequencing of the putative DNA helicase-encoding gene of the *Bombyx mori* nuclear polyhedrosis virus and fine mapping of a region involved in host range expansion. *Gene* **190**, 173-179.

Kang, W., Tristem, M., Maeda, S., Crook, N. E. and O'Reilly, D. R. (1998). Identification and characterization of the *Cydia pomonella* granulovirus cathepsin and chitinase genes. *Journal of General Virology* **79**, 2283-2292.

King, L. A. and Possee, R. D. (1992). *The Baculovirus System: A Laboratory Guide*. London: Chapman and Hall.

Knebel-Mörsdorf, D., Flipsen, J. T. M., Roncarati, R., Jahnel, F., Kleefsman, A. W. F. and Vlak, J. M. (1996). Baculovirus infection of *Spodoptera exigua* larvae: *lacZ* expression driven by promoters of early genes *pe38* and *me53* in larval tissue. *Journal of General Virology* **77**, 815-824.

Kogan, P. H., Chen, X. Y. and Blissard, G. W. (1995). Overlapping TATA-dependent and TATA-independent early promoter activities in the baculovirus *gp64* envelope fusion protein gene. *Journal of Virology* **69**, 1452-1461.

Kondo, A. and Maeda, S. (1991). Host range expansion by recombination of the baculoviruses *Bombyx mori* nuclear polyhedrosis virus and *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **65**, 3625-3632.

Kool, M., Ahrens, C. H., Goldbach, R. W., Rohrmann, G. F. and Vlak, J. M. (1994*a*). Identification of genes involved in DNA-replication of the *Autographa californica* baculovirus. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11212-11216.

Kool, M., Ahrens, C. H., Vlak, J. M. and Rohrmann, G. F. (1995). Replication of baculovirus DNA. *Journal of General Virology* **76**, 2103-2118.

Kool, M., Voeten, J. T. M., Goldbach, R. W. and Vlak, J. M. (1994*b*). Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis viral genome required for DNA-replication. *Virology* **198**, 680-689.

Kramer, K. J. and Muthukrishnan, S. (1997). Insect chitinases: Molecular biology and potential use as biopesticides. *Insect Biochemistry and Molecular Biology* **27**, 887-900.

Krappa, R., Behnkrappa, A., Jahnel, F., Doerfler, W. and Knebel-Mörsdorf, D. (1992). Differential factor binding at the promoter of early baculovirus gene *pe38* during viral infection - GATA motif is recognized by an insect protein. *Journal of Virology* **66**, 3494-3503.

Kuzio, J., Jaques, R., Faulkner, P. (1989). Identification of p74, a gene essential for virulence of baculovirus occlusion bodies. *Virology* **173**, 759-763.

Kuzio, J., Pearson, M. N., Harwood, S. H., Funk, C. J., Evans, J. T., Slavicek, J. M. and Rohrmann, G. F. (1999). Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology* **253**, 17-34.

Leisy, D. J. and Rohrmann, G. F. (1993). Characterization of the replication of plasmids containing *hr* sequences in baculovirus infected *Spodoptera frugiperda* cells. *Virology* **196**, 722-730.

Lepore, L. S., Roelvink, P. R. and Granados, R. R. (1996). Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloprotease. *Journal of Invertebrate Pathology* **68**, 131-140.

Léry, X., El-Tarras, A., Abol-Ela, S. and Giannotti, J. (1998). Yield and activity of *Autographa californica* multicapsid nucleopolyhedrovirus and *Phthorimaea operculella* granulosis virus in cloned and uncloned cell lines of *P. operculella*. *CYTOTECHNOLOGY* **26**, 103-110

Léry X, Giannotti J, Taha, A., Ravalec, M. and Abol-Ela, S. (1997). Multiplication of a granulosis virus isolated from the potato tuber moth in a new established cell line of *Phthorimaea operculella*. *IN VITRO CELL DEV-AN* **33**, 640-646

Li, Y. H. and Miller, L. K. (1995). Properties of a baculovirus mutant defective in the protein phosphatase gene. *Journal of Virology* **69**, 4533-4537.

Lovelidge, B. (1997). New weapon in the tortrix war. *Grower* **127**, 33.

Lu, A. and Miller, L. K. (1995). The roles of 18 baculovirus late expression factor genes in transcription and DNA-replication. *Journal of Virology* **69**, 975-982.

Lu, A. and Miller, L. K. (1997). Regulation of baculovirus late and very late gene expression. In *The Baculoviruses*, pp. 193-216. Edited by L. K. Miller. New York: Plenum Press.

Maeda, S. (1989*a*). Expression of foreign genes in insects using baculovirus vectors. *Annual Review of Entomology* **34**, 351-372.

Maeda, S. (1989*b*). Increased insecticidal effect by a recombinant baculovirus carrying a synthetic diuretic hormone gene. *Biochemical and Biophysical Research Communications* **165**, 1177-1183.

Maeda, S., Kamita, S. G. and Kondo, A. (1993). Host range expansion of *Autographa californica* nuclear polyhedrosis virus (NPV) following recombination of a 0.6 -kilobase-pair DNA fragment originating from *Bombyx mori* NPV. Journal of Virology **71**, 1851-1855.

Maeda, S., Volrath, S. L., Hanzlik, T. N., Harper, S. A., Kei, M. M., Maddox, D. W., Hammock, B. D. and Fowler, E. (1991). Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. *Virology* **184**, 777-780.

McClintock, J. T. and Dougherty, E. M. (1987). Superinfection of baculovirus infected gypsy moth cells with the nuclear polyhedrosis viruses *of Autographa californica* and *Lymantria dispar*. *Virus Research* **7**, 351-364.

McLachlin, J. R. and Miller, L. K. (1994). Identification and characterization of *vlf-1*, a baculovirus gene involved in very late gene-expression. *Journal of Virology* **68**, 7746-7756.

Mikhailov, V. S., Marlyev, K. A., Ataeva, J. O., Kullyev, P. K. and Atrazhev, A. M. (1986). Characterization of 3'-5' exonuclease associated with DNA-polymerase of silkworm nuclear polyhedrosis virus. *Nucleic Acids Research* **14**, 3841-3857.

Miller, L. K. (1995). Genetically engineered insect virus pesticides - present and future. *Journal of Invertebrate Pathology* **65**, 211-216.

Miller, L. K. (1997). The Baculoviruses. In *The Viruses*. Edited by H. Fraenkel-Conrat and R. R. Wagner. New York: Plenum press.

Miller, L. K., Jewell, J. E. and Browne, D. (1981). Baculovirus induction of a DNA-polymerase. *Journal of Virology* **40**, 305-308.

Miller, L. K. and Lu, A. (1997). The molecular basis of baculovirus host range. In *The Baculoviruses*, pp. 217-235. Edited by L. K. Miller. New York: Plenum Press.

Miller, D. W. and Miller, L. K. (1982). A virus mutant with an insertion of a copia-like transposable element. *Nature* **299**, 562-564.

Miltenburger, H. G., Naser, W. L., Harvey, J. P., Huber, J. and Huger, A. M. (1984). The cellular substrate - a very important requirement for baculovirus *in vitro* replication. *Zeitschrift Fur Naturforschung - a Journal of Biosciences* **39**, 993-1002.

Morris, T. D. and Miller, L. K. (1992). Promoter influence on baculovirus mediated gene expression in permissive and nonpermissive insect cell lines. *Journal of Virology* **66**, 7397-7405.

Moscardi, F. (1999). Assessment of the application of baculoviruses for control of Lepidoptera. *Annual Review of Entomology* **44**, 257-289.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., Summers, M. D. (1995). Virus taxonomy - classification and nomenclature of viruses. Springer-Verlag/Wien: Springer-Verlag Wien New York.

Mussche, G. and Garbous, B. (1986). La luttle integre contre le carpocapse, *Laspeyresia pomonella*, des abres a pepins dans le nord de la Tunisie mededelingen van de Faculteit land bouwwetenschappen. *Rijksuniversiteit Gent* **51**, 1095-1102.

Naser, W. L., Miltenburger, H. G., Harvey, J. P., Huber, J. and Huger, A. M. (1984). *In vitro* replication of the *Cydia pomonella* (Codling Moth) granulosis virus. *Fems Microbiology Letters* **24**, 117-121.

Nishi, Y. and Nonaka, T. (1996). Biological control of the tea tortrix. *Agrochem. Jpn.* **69**, 7-10.

Ohkawa, T., Majima, K. and Maeda, S. (1994). A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. *Journal of Virology* **68**, 6619-6625.

Ohresser, M., Morin, N., Cerutti, M. and Delsert, C. (1995). Sequence analysis and transcriptional mapping of the *orf-2* gene of *Autographa californica* nuclear polyhedrosis virus. *Gene* **152**, 201-204.

Oppenheimer, D. I. and Volkman, L. E. (1997). Evidence for rolling circle replication of *Autographa californica* M nucleopolyhedrovirus genomic DNA. *Archives of Virology* **142**, 2107-2113.

O'Reilly, D. (1997). Auxiliary genes of baculoviruses. In *The Baculoviruses*, pp. 267-300. Edited by L. K. Miller. New York: Plenum Press.

O'Reilly, D. R., Brown, M. R. and Miller, L. K. (1992). Alteration of ecdysteroid metabolism due to baculovirus infection of the fall armyworm *Spodoptera frugiperda* - host ecdysteroids are conjugated with galactose. *Insect Biochemistry and Molecular Biology* **22**, 313-320.

O'Reilly, D. R., Kelly, T. J., Masler, E. P., Thyagaraja, B. S., Robson, R. M., Shaw, T. C. and Miller, L. K. (1995). Overexpression of *Bombyx mori* prothoracicotropic hormone using baculovirus vectors. *Insect Biochemistry and Molecular Biology* **25**, 475-485.

O'Reilly, D. R. and Miller, L. K. (1989). A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. *Science* **245**, 1110-1112.

O'Reilly, D. R. and Miller, L. K. (1990). Regulation of expression of a baculovirus ecdysteroid UDP-glucosyltransferase gene. *Journal of Virology* **64**, 1321-1328.

O'Reilly, D. R. and Miller, L. K. (1991). Improvement of a baculovirus pesticide by deletion of the EGT gene. *Bio-Technology* **9**, 1086-1089.

O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1992). Baculovirus expression vectors. New York: Freeman.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comp. Appl. Biosci.* **12**, 357-358.

Pardini, R. S. (1995). Toxicity of oxygen from naturally-occurring redox-active pro-oxidants. *Archives of Insect Biochemistry and Physiology* **29**, 101-118.

Possee, R. D. (1997). Baculoviruses as expression vectors. *Current Opinion in Biotechnology* **8**, 569-572.

Possee, R. D., Barnett, A. L., Hawtin, R. E. and King, L. A. (1997). Engineered baculoviruses for pest control. *Pesticide Science* **51**, 462-470.

Possee, R. D. and Rohrmann, F. (1997). Baculovirus Genome Organization and Evolution. In *The Baculoviruses*, pp. 109-140. Edited by L. K. Miller. New York: Plenum Press.

Possee, R. D., Sun, T. P., Howard, S. C., Ayres, M. D., Hillperkins, M. and Gearing, K. L. (1991). Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis 9.4 kbp *Eco*RI-I and *Eco*RI-R (polyhedrin gene) region. *Virology* **185**, 229-241.

Pullen, S. S. and Friesen, P. D. (1995). The CAGT motif functions as an initiator element during early transcription of the baculovirus transregulator *ie-1*. *Journal of Virology* **69**, 3575-3583.

Reilly, L. M. and Guarino, L. A. (1994). The *pk-1* gene of *Autographa californica* multinucleocapsid nuclear polyhedrosis virus encodes a protein kinase. *Journal of General Virology* **75**, 2999-3006.

Reilly, L. M., Guarino, L. A. (1996). The viral ubiquitin gene of *Autographa californica* nuclear polyhedrosis virus is not essential for viral replication. *Virology* **218**, 243-247.

Reynolds, D. S. (1963). The use of lead citrate at a high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208.

Richards, A., Matthews, M. and Christian, P. (1998). Ecological considerations for the environmental impact evaluation of recombinant baculovirus insecticides. *Annual Review of Entomology* **43**, 493-517.

Riegel, C. L., Lanner-Herrera, C. and Slavicek, J. M. (1994). Identification and characterization of the ecdysteroid UDP-glucosyltransferase gene of the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. *Journal of General Virology* **75**, 829-838.

Ripa, R. (1982). Field evaluation of a granulosis virus for the control of the codling moth. *Invertebrate Pathology and Microbial control*, 3rd International Colloquium on Invertebrate Pathology, 6-10 September, University of Sussex, Brighton, UK.

Roelvink, P. W., Corsaro, B. G. and Granados, R. R. (1995). Characterization of the *Helicoverpa armigera* and *Pseudaletia unipuncta* granulovirus enhancin genes. *Journal of General Virology* **76**, 2693-2705.

Russell, R. L. Q., Funk, C. J. and Rohrmann, G. F. (1997). Association of a baculovirus encoded protein with the capsid basal region. *Virology* **227**, 142-152.

Russell, P. and Nurse, P. (1987). Negative regulation of mitosis by *wee1+*, a gene encoding a protein kinase homolog. *Cell* **49**, 559-567.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning. A laboratory manual: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen and Coulsen, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Acadamy of Sciences USA*, **74**, 5463-7.

Schaffer, H.E and R.R. Sederoff. (1981). Improved estimation of DNA fragment length from agarose gels. *Anal. Biochem.* **115**,113-122.

Schmid, A. (1983). A granulosis virus of the fruit tortrix, *Adoxophyes orana* F. v. R. (Lep., Tortricidae). *Bulletin de la Societe Entomologique Suisse* **56**, 225-235.

Slack, J. M., Kuzio, J. and Faulkner, P. (1995). Characterization of v-cath, a cathepsin l-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis-virus. *Journal of General Virology* **76**, 1091-1098.

Smith, G. E., Summers, M. D. and Fraser, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* **3**, 2156-2165.

Smith, I. R. L. (1987). Genome structure and host interactions of *Pieris* granulosis viruses. In *Department of Entomology and Insect Pathology*. Institute of Horticultural Research, Littlehampton: London.

Smith, I. R. L. and Crook, N. E. (1988). *In vivo* isolation of baculovirus genotypes. *Virology* **166**, 240-244.

Smith, I. R. L. and Goodale, C. (1998). Sequence and *in vivo* transcription of *Lacanobia oleracea* granulovirus EGT. *Journal of General Virology* **79**, 405-413.

Smith, O. J., Hughes, K. M., Dunn, P. H. and Hall, I. M. (1956). A granulosis virus disease of the Western grapeleaf skeletonizer and its transmission. *Can. Entomol.* **88**, 507.

Sokal, R.R. and Rohlf, F.J. (1995) Biometry: The principles and practice of statistics in biological research (Third Edition). W.H.Freeman & Co., New York.

Solomon, M. G. (1982). Phytophagous mites and their predators in apple orchards. *Annals of Applied Biology* **101**, 201-203.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-507.

Spurr, A. R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of ultrastructure Research* **26**, 31-34.

Subinprasert, S. and Svensson, B. W. (1988). Effects of predation on clutch size and egg dispersion in the codling moth *Laspeyresia pomonella*. *Ecological Entomology* **13**, 87-94.

Summers, M. D. (1971). Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of *Trichoplusia ni*. *Journal of Ultrastructure Research* **35**, 606-625.

Tanada, Y. (1964). A granulosis virus of codling moth, *Carpocapsa pomonella* (linnaeus) (Olethreutidae, Lepidoptera). *Journal of Insect Pathology* **6**, 378-380.

Theilmann, D. A. and Stewart, S. (1992). Tandemly repeated sequence at the 3' end of the *ie-2* gene of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus is an enhancer element. *Virology* **187**, 97-106.

Thiem, S. M., Du, X. L., Quentin, M. E. and Berner, M. M. (1996). Identification of a baculovirus gene that promotes *Autographa californica* nuclear polyhedrosis virus replication in a nonpermissive insect cell line. *Journal of Virology* **70**, 2221-2229.

Thomas, C. J, Brown, H. L., Hawes, C. R., Lee, B. Y., Min, M. K., King, L.A., Possee, R. D. (1998). Localization of a baculovirus induced chitinase in the insect cell endoplasmic reticulum. *Journal of Virology* **72**, 10207-10212.

Thomas, G. M. and Poinar, G. O. (1973). Report of diagnoses of diseased insects 1962-1972. *Hilgardia* **42**, 261-360.

Todd, J. W., Passarelli, A. L. and Miller, L. K. (1995). 18 baculovirus genes, including *lef-11, P35, 39k* and *p47*, support late gene expression. *Journal of Virology* **69**, 968-974.

Tomalski, M. D., Bruce, W. A., Travis, J. and Blum, M. S. (1988a). Preliminary characterization of toxins from the straw itch mite, *Pyemotes tritici*, which induce paralysis in the larvae of a moth. *Toxicon* **26**, 127-132.

Tomalski, M. D., Kutney, R., Bruce, W. A., Brown, M. R., Blum, M. S. and Travis, J. (1989). Purification and characterization of insect toxins derived from the mite, *Pyemotes tritici*. *Toxicon* **27**, 1151-1167.

Tomalski, M. D., Wu, J. G. and Miller, L. K. (1988b). The location, sequence, transcription, and regulation of a baculovirus DNA polymerase gene. *Virology* **167**, 591-600.

Tomalski, M. D., Eldridge, R., Miller, L. K. (1991). A baculovirus homolog of a cu/zn superoxide-dismutase gene. *Virology* **184**, 149-161.

van Oers, M. M. and Vlak, J. M. (1997). The baculovirus 10-kDa protein. *Journal of Invertebrate Pathology* **70**, 1-17.

van Beek, N. A. M. and Hughes, P. R. (1986). Determination by fluorescence spectroscopy of the volume ingested by neonate lepidopterous larvae. *Journal of Invertebrate Pathology* **48**, 249-251.

Vialard, J. E., Arif, B. M. and Richardson, C. D. (1995). Introduction to the Molecular Biology of Baculoviruses. In *Methods in Molecular Biology, Vol 39: Baculovirus Expression Protocols*, pp.1-23. Edited by C. D. Richardson. Totowa, NJ: Human Press Inc.

Vlak, J. M., Schouten, A., Usmany, M., Belsham, G. J., Klingeroode, E. C., Maule, A. J., Vanlent, J.W.M and Zuidema, D. (1990). Expression of cauliflower mosaic-virus gene-1 using a baculovirus vector based upon the *p10* gene and a novel selection method. *Virology* **179**, 312-320.

Wang, P., Hammer, D. A. and Granados, R. R. (1994). Interaction of *Trichoplusia ni* granulosis virus-encoded enhancin with the midgut epithelium and peritrophic membrane of 4 lepidopteran insects. *Journal of General Virology* **75**, 1961-1967.

Wang, X. and Kelly, D. C. (1983). Baculovirus replication - purification and identification of the *Trichoplusia ni* nuclear polyhedrosis virus-induced DNA-polymerase. *Journal of General Virology* **64**, 2229-2336.

Ward, V. K., Bonning, B. C., Huang, T., Shiotsuki, T., Griffeth, V. N. and Hammock, B. D. (1992). Analysis of the catalytic mechanism of juvenile hormone esterase by site-directed mutagenesis. *International Journal of Biochemistry* **24**, 1933-1941.

Washburn, J. O., Kirkpatrick, B. A. and Volkman, L. E. (1996). Insect protection against viruses. *Nature* **383**, 767.

Whitford, M., Stewart, S., Kuzio, J. and Faulkner, P. (1989). Identification and sequence analysis of a gene encoding *gp67* an abundant envelope glycoprotein of the baculovirus, *Autographa californica* nuclear polyhedrosis virus, *J. virol.* **63**, 1393.

Wilson, L. E., Wilkinson, N., Marlow, S. A., Possee, R. D. and King, L. A. (1997). Identification of recombinant baculoviruses using green fluorescent protein as a selectable marker. *Biotechniques* **22**, 674.

Wilson, M. E. and Consigli, R. A. (1985*a*). Characterization of a protein kinase activity associated with purified capsids of the granulosis virus infecting *Plodia interpunctella*. *Virology* **143**, 516-525.

Wilson, M. E. and Consigli, R. A. (1985*b*). Functions of a protein kinase activity associated with purified capsids of the granulosis virus infecting *Plodia interpunctella*. *Virology* **143**, 526-535.

Winstanley, D. and Crook, N. E. (1993). Replication of *Cydia pomonella* granulosis virus in cell cultures. *Journal of General Virology* **74**, 1599-1609.

Wood, H. A. and Granados, R. R. (1991). Genetically engineered baculoviruses as agents for pest-control. *Annual Review of Microbiology* **45**, 69-87.

Wood, H. A. and Hughes, P. R. (1993). Biopesticides. *Science* **261**, 277-277.

Xie, W. D., Arif, B., Dobos, P. and Krell, P. J. (1995). Identification and analysis of a putative origin of DNA replication in the *Choristoneura fumiferana* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* **209**, 409-419.

Yamada, H. and Ono, N. (1973). A granulosis virus, possible biological agent for control of *Adoxophyes orana* (Lepidoptera: Tortricidae) in apple orchards. *Journal of Invertebrate Pathology* **21**, 144-148.

Yang, C. L., Stetler, D. A. and Weaver, R. F. (1991). Structural comparison of the Autographa californica nuclear polyhedrosis virus-induced RNA-polymerase and the 3 nuclear-RNA polymerases from the host, *Spodoptera frugiperda. Virus Research* **20**, 251-264.

Yang, S. and Miller, L. K. (1999). Activation of baculovirus very late promoters by interaction with very late factor 1. *Journal of Virology* **73**, 3404-3409.

Zhang, C. X., Hu, C. and Wu, X. F. (1998). Studies on the nucleotide sequence, transcription and deletion analysis of the BmNPV protein kinase gene. *Acta Biochimica Et Biophysica Sinica* **30**, 185-190.

Zlotkin, E., Rochat, H., Kopeyan., C., Miranda, F. and Lissitzky, S. (1971). Purification and properties of the insect toxin from the venom of the scorpion *Androctonus australis* Hector. *Biochimie* **53**, 1073-1078.

Zuidema, D., Schouten, A., Usmany, M., Maule, A. J., Belsham, G. J., Roosien, J., Klinge-Roode, E. C., van Lent, J. W. M. and Vlak, J. M. (1990). Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. *Journal of General Virology* **71**, 2201-2209.

APPENDIX 1

Artificial diet for codling moth (*C. pomonella*), false codling moth (*C. leucotreta*) and summer fruit tortrix moth (*A. orana*) larvae.

Maize meal	380 g
Yeast powder	100 g
Wheat germ	92 g
Ascorbic acid	14 g
Benzoic acid	6 g
10 % Formaldehyde	9.6 ml
Methyl parahydroxybenzoate	4.6 g
Agar	53.2 g

1. Dissolve agar in 1200 ml water. Dissolve in a microwave.
2. Mix dry ingredients in a blender. Add remaining 800 ml water and formaldehyde.
3. Add agar after cooling to 70 °C.
4. Stir and then dispense in a tray and leave to solidify.

a) For diet used for infecting larvae, formaldehyde is omitted from the diet.

b) When juvenile hormone is required 2 ml of a 0.003 working stock of methoprene is added.

c) 'Water diet' used for neonate bioassays in 96 well plates contain an extra 330 ml water.

APPENDIX 2

IZD04 MEDIUM

STOCK SOLUTIONS

Soluble amino acid stock solutions (:25X) (all Sigma, cell culture grade)

	g		g
L-arginine x HCl	17.5	L-isoleucine	1.25
L-aspartic acid	8.75	L-leucine	1.88
L-asparagine	8.75	L-lysine x HCl	15.63
L-alanine	5.63	L-methionine	1.25
β-alanine	5.0	L-proline	8.75
Glutamic acid	15.0	L-phenylalanine	3.75
Glutamine	15.0	DL-serine	27.5
Glycine	16.0	L-threonine	4.38
L-histidine x HCl	62.5	L-valine	2.5

Dissolve in 1 litre Milli-Q water

Store in 200 ml aliquots at –20°C

Insoluble amino acid stock solutions (25X) (Sigma, cell culture grade)

	g
L-cystine	0.55
L-tryptophan	2.5
L-tyrosine	1.25

Dissolve in 900 ml water. Add 2N HCl to aid dissolving.

Make up to 1 litre.

Store at –20°C

Vitamin stock solution (100X) (Sigma, cell culture grade)

	mg
p-aminobenzoic acid	2
Biotin	0.5
D-Ca-Pantothenate	2
Choline chloride	10
Folic acid	2
i-inositol	2
Nicotinic acid	2
Pyridoxine x HCl	2
Thiamine x HCl	2

Dissolve in 1 litre Milli-Q water.

Aliquot in 50 ml quantities.

Store at –70°C.

To make up 5 litre of IZDO4 medium

Combine in order with stirring

1. 200 ml soluble amino acids (25X)
2. 400 ml Milli-Q water
3. 50 ml vitamin stock solution (100X)
4. 200 ml insoluble amino acids (25X)
5. 13 g bactotryptose (Difco)
6. 5 g glucose
7. 1 litre **Solution A**

	g
KCl	14.35
$CaCl_2.2H_2O$	3.3
$MgCl_2.6H_2O$	5.7
$MgSO_4.7H_2O$	6.976

(The $MgCl_2.6H_2O$ must be dissolved before the $MgSO_4.7H_2O$ is added)

8. 100 ml **Solution B**

	g
NaH_2PO_4	2.192
$NaHCO_3$	1.75

pH 6.3 with 2N HCl

9. Dilute with Milli-Q water and adjust pH to 6.4 with 1N KOH. Make up to 4.5 litres. Filter sterilize (0.2 μm filter).

10. Foetal bovine serum (FBS) is added to 100-200 ml quantities at 10 % to make up complete medium.

11. Medium is stored at 4°C.

APPENDIX 3

APPENDIX TABLE 3.1

Fragments of ClGV DNA that hybridised to CpGV-M1 cosmids. Two fragment letters adjacent without a comma between indicates comigrating fragments where the actual hybridising fragment has not been determined.

CpGV cosmid	ClGV *Bam*HI fragments hybridising	ClGV *Eco*RI fragments hybridising	ClGV *Kpn*I fragments hybridising	ClGV *Nde*I fragments hybridising
M73	A, H, J, M	A, C, L	A, B, I, J,	D, F, K, L
M64	C, F, H	A, E	A	E, H, I, K
M65	C, D,K, I	D, E, F, K	A, C, G, H, K	A, I
M17	D, G	D, K, N	C, F	A, B
M69	B, E	B, G	D, E, F	B
M2	A	C, I, J, LM, P, R,	B, E	C, D, G, N

APPENDIX TABLE 3.2

Fragments of PrGV DNA that hybridised to CpGV-M1 cosmids.

CpGV cosmid	PrGV *Bam*HI fragments hybridising	PrGV *Eco*RI fragments hybridising	PrGV *Hind*III fragments hybridising	PrGV *Kpn*I fragments hybridising
M73	D, F, H, M	D, F, H, J, P	B, C, E	D, G, J, M, N
M64	DE	D, J, O	C, E, H	D, E
M65	C, K	C, E, R	A	A, H
M17	C, K, G	B, C, E	A	A, H
M69	B, A	A, B, G, L	B, D, G, K	B, I
M2	A, F	A, H, P	B, C	C, G, I

APPENDIX TABLE 3.3

Fragments of PxGV DNA that hybridised to CpGV-M1 cosmids. Two fragment letters adjacent without a comma between indicates comigrating fragments where the actual hybridising fragment has not been determined.

CpGV cosmid	PxGV *Bam*HI fragments hybridising	PxGV *Eco*RI fragments hybridising	PxGV *Pst*I fragments hybridising
M73	A, D, G	A, B	D, E, G, MN
M64	B, D	A	B, I
M65	B, C	C, D, H	A, B, H, L
M17	B, F	D, G	FH, L, R
M69	C, E,	C, F	A, Q
M2	A	B, E	C, D, J

APPENDIX TABLE 3.4

Fragments of LoGV DNA that hybridised to CpGV-M1 cosmids. Two fragment letters adjacent without a comma between indicates comigrating fragments where the actual hybridising fragment has not been determined.

CpGV cosmid	LoGV *Pst*I fragments hybridising	LoGV *Xho*I fragments hybridising
M73	A, D, GH, I, K, L, PQ	BC, D, M
M64	A, D, G	E, H, M, N
M65	D, F, J, K	DE, J, K, L,O
M17	B	F, L, S
M69	C, E, M	A, G
M2	H, L, N	B, C, I

APPENDIX TABLE 3.5

Fragments of AoGV DNA that hybridised to CpGV-M1 cosmids.

CpGV cosmid	AoGV *Bam*HI fragments hybridising	AoGV *Bgl*II fragments hybridising	AoGV *Pst*I fragments hybridising	AoGV *Sac*I fragments hybridising
M73	B, F, G	A	A	B
M64	D, F	A	A	C
M65	C	C	D, E, F	A, C
M17	C, E	C, D	C, D	A
M69	A,	B, E, F	B	A, B
M2	A, B	A, B, H, I	A, B	B

APPENDIX TABLE 3.6

Fragments of TnGV DNA that hybridised to CpGV-M1 cosmids. Two fragment letters adjacent without a comma between indicates comigrating fragments where the actual hybridising fragment has not been determined.

CpGV cosmid	TnGV *Bam*HI fragments hybridising	TnGV *Eco*RI fragments hybridising	TnGV *Pst*I fragments hybridising	TnGV *Xho*I fragments hybridising
M73	B, G, IJ	A, F, I, S	_____	A, C, G
M64	A, J	I, O	J, W	A, D
M65	K, M	M	Q	L
M17	A, K, M	B, M	B, Q	B, L
M69	A	D, J	G, H	_____
M2	B, F, H	A, K	A, C, F, P	E, G, J, K

APPENDIX TABLE 3.7

Fragments of XcGV DNA that hybridised to CpGV-M1 cosmids.

CpGV cosmid	XcGV *Bam*HI fragments hybridising	XcGV *Bgl*II fragments hybridising	XcGV *Eco*RI fragments hybridising
M73	A, D, H, I, J, K, N	B, D, E, G	A, C, G, L, Q
M64	B, C, J, K, L	A, D, E	D, G, M, Q
M65	E	A	B
M17	B, E	A	B, E, R
M69	A, F, K	C, E	F, G, N
M2	A	C, G, L	A, J

APPENDIX TABLE 3.8

Fragments of XcGV DNA that hybridised to fragments of TnGV DNA. Two fragment letters adjacent without a comma between indicates comigrating fragments where the actual hybridising fragment has not been determined.

TnGV fragments	XcGV *Bam*HI fragments hybridising	XcGV *Bgl*II fragments hybridising	XcGV *Eco*RI fragments hybridising
*Pst*I A, C	A, D, G, I	B, G, H	A, C, H
*Bam*HI C, D, G	C, E, H	D, F, K	B, D, L
*Xho*I L, A	B, E	A	B, E, K, M, P, R
*Bam*HI A	A, B, F, J, K, M	A, C, E	EF, G, I, N, O, Q
*Eco*RI A, D	A	C, G, H, I, L	A, F, J, S

APPENDIX TABLE 3.9

Fragments of ClGV DNA that hybridised to fragments of TnGV DNA.

TnGV fragments	ClGV *Bam*HI fragments hybridising	ClGV *Eco*RI fragments hybridising	ClGV *Kpn*I fragments hybridising	ClGV *Nde*I fragments hybridising
*Pst*I A, C	A	C, L	B, J	D, F
*Bam*HI C, D, G	F	A	A	E
*Xho*I L, A	———	———	———	———
*Bam*HI A	B, E	B, G	D	B
*Eco*RI A, D	A	C, R	B	C, D

APPENDIX TABLE 3.10

Fragments of AoGV DNA that hybridised to fragments of TnGV DNA.

TnGV fragments	AoGV *Bam*HI fragments hybridising	AoGV *Bgl*II fragments hybridising	AoGV *Pst*I fragments hybridising	AoGV *Sac*I fragments hybridising
*Pst*I A, C	B	A	A	B
*Bam*HI C, D, G	D	A	A	C
*Xho*I L, A	C	C	D	A
*Bam*HI A	A, E	D, E	B, C	A
*Eco*RI A, D	A, B	A, B	A, B	B

APPENDIX 4

APPENDIX TABLE 4.1

Neonate bioassay LD_{50} data

	BIOASSAY 1			BIOASSAY 2			BIOASSAY 3		
Dose (OBs)	No. infected	No. dead	% dead	No. infected	No. dead	% dead	No. infected	No. dead	% dead
5	45	6	13.33	39	4	10.26	42	5	11.90
15	42	11	26.19	44	14	31.82	46	11	23.91
45	42	25	59.52	43	22	51.16	39	30	76.92
135	42	37	88.10	44	35	79.55	42	35	83.33
405	45	44	97.78	46	46	100.0	38	37	97.37

APPENDIX TABLE 4.2

Fourth instar bioassay LD_{50} data

	BIOASSAY 1			BIOASSAY 2			BIOASSAY 3		
Dose (OBs)	No. infected	No. dead	% dead	No. infected	No. dead	% dead	No. infected	No. dead	% dead
$2x10^2$	39	4	10.26	49	4	81.63	45	1	2.22
$1x10^3$	37	11	29.73	47	5	10.64	49	17	34.69
$5x10^3$	43	28	65.12	47	11	23.40	45	19	42.22
$2.5x10^4$	33	27	81.82	46	32	69.57	40	28	70.00
$1.25x10^5$	38	36	94.74	44	35	79.55	41	39	95.12

APPENDIX TABLE 4.3

Fifth instar bioassay LD_{50} data

	BIOASSAY 1			BIOASSAY 2			BIOASSAY 3		
Dose (OBs)	No. infected	No. dead	% dead	No. infected	No. dead	% dead	No. infected	No. dead	% dead
$5x10^4$	49	10	20.41	50	6	12.00	47	13	27.66
$4x10^5$	45	18	40.00	50	13	26.00	49	14	28.57
$3.2x10^6$	48	30	62.50	50	26	52.00	46	25	54.35
$2.56x10^7$	46	39	84.78	48	38	79.17	49	44	89.80
$2x10^8$	49	44	89.80	50	49	98.00	42	39	92.86

APPENDIX TABLE 4.4

Neonate ST_{50} data

	BIOASSAY (no. dead)			BIOASSAY (% of infected larvae dead)			
d. p. i	1	2	3	1	2	3	mean
21	1	0	0	2.7	0	0	0.9
22	1	0	0	5.4	0	0	1.8
23	0	0	0	5.4	0	0	1.8
24	0	0	1	5.4	0	3.0	2.8
25	0	1	0	5.4	2.1	3.0	3.5
26	0	0	0	5.4	2.1	3.0	3.5
27	0	2	0	5.4	6.4	3.0	4.9
28	1	2	0	8.1	10.6	3.0	7.3
29	0	1	1	8.1	12.8	6.1	9.0
30	0	0	0	8.1	12.8	6.1	9.0
31	1	1	1	10.8	14.9	9.1	11.6
32	1	2	2	13.5	19.1	15.2	15.9
33	3	3	1	21.6	23.4	18.2	21.1
34	4	3	3	32.4	29.8	27.3	29.8
35	4	3	1	43.2	36.2	30.3	36.6
36	1	7	4	45.9	51.1	42.4	46.5
37	2	5	2	51.4	55.3	48.5	51.7
38	0	3	0	51.4	61.7	48.5	53.8
39	3	5	2	59.5	66.0	54.5	60.0
40	0	1	0	59.5	68.1	54.5	60.7
41	1	2	4	62.2	72.3	66.7	67.1
42	2	0	0	67.6	72.3	66.7	68.9
43	1	1	2	70.3	78.7	72.7	73.9
44	3	0	2	78.4	83.0	78.8	80.0
45	1	0	1	81.1	83.0	81.8	82.0
46	2	1	1	86.5	85.1	84.8	85.5
47	0	1	1	86.5	87.2	87.9	87.2
48	0	1	1	86.5	87.2	90.9	88.2
49	0	0	0	86.5	89.4	90.9	88.9
50	1	0	0	89.2	93.6	90.9	91.2
51	0	0	0	89.2	93.6	90.9	91.2
52	2	0	1	94.6	95.7	93.9	94.8
53	0	1	0	94.6	97.9	93.9	95.5
54	0	1	1	94.6	100.0	97.0	97.2
55	0	0	0	94.6	100.0	97.0	97.2
56	1	0	1	97.3	100.0	100.0	99.1
57	0	0	0	97.3	100.0	100.0	99.1
58	1	0	0	100.0	100.0	100.0	100.0
Total dead	37	47	33				
No. in assay	54	64	42				
% dead	68.5	73.4	78.6				

APPENDIX TABLE 4.5

Fourth instar ST_{50} data

	BIOASSAY (no. dead)			BIOASSAY (% of infected larvae dead)			
d. p. i	1	2	3	1	2	3	mean
17	1	0	0	1.4	0.0	0.0	0.5
18	0	1	0	1.4	1.5	0.0	1.0
19	0	1	0	1.4	3.1	0.0	1.5
20	1	0	1	2.9	3.1	2.1	2.7
21	3	7	1	7.2	13.8	4.3	8.4
22	4	0	.3	13.0	13.8	10.6	12.5
23	3	0	1	17.4	13.8	12.8	14.7
24	0	2	1	17.4	16.9	14.9	16.4
25	7	6	2	27.5	26.2	19.1	24.3
26	2	3	3	30.4	30.8	25.5	28.9
27	11	6	6	46.4	40.0	38.3	41.6
28	2	3	2	49.3	44.6	42.6	45.5
29	5	4	1	56.5	50.8	44.7	50.7
30	2	6	8	59.4	60.0	61.7	60.4
31	4	6	3	65.2	69.2	68.1	67.5
32	5	4	3	72.5	75.4	74.5	74.1
33	5	2	1	79.7	78.5	76.6	78.3
34	4	3	2	85.5	83.1	80.9	83.1
35	5	4	1	92.8	89.2	83.0	88.3
36	1	1	1	94.2	90.8	85.1	90.0
37	1	0	3	95.7	90.8	91.5	92.6
38	1	1	1	97.1	92.3	93.6	94.3
39	0	1	1	97.1	93.8	95.7	95.6
40	1	1	0	98.6	95.4	95.7	96.6
41	0	1	0	98.6	96.9	95.7	97.1
42	1	0	0	100.0	96.9	95.7	97.6
43	0	1	0	100.0	98.5	95.7	98.1
44	0	1	0	100.0	100.0	95.7	98.6
45	0	0	0	100.0	100.0	95.7	98.6
46	0	0	0	100.0	100.0	95.7	98.6
47	0	0	0	100.0	100.0	95.7	98.6
48	0	0	0	100.0	100.0	95.7	98.6
49	0	0	0	100.0	100.0	95.7	98.6
50	0	0	0	100.0	100.0	95.7	98.6
51	0	0	0	100.0	100.0	95.7	98.6
52	0	0	0	100.0	100.0	95.7	98.6
53	0	0	0	100.0	100.0	95.7	98.6
54	0	0	1	100.0	100.0	97.9	99.3
55	0	0	1	100.0	100.0	100.0	100.0
Total dead	69	65	47				
No. in assay	71	73	55				
% dead	97.2	89.0	85.5				

APPENDIX TABLE 4.6

Fifth instar ST_{50} data

	BIOASSAY (no. dead)			BIOASSAY (% of infected larvae dead)			
d. p. i	1	2	3	1	2	3	mean
15	0	1	1	0	1.4	1.6	1.0
16	0	0	1	0	1.4	3.1	1.5
17	0	0	1	0	1.4	4.7	2.0
18	0	5	3	0	8.5	9.4	5.9
19	1	2	1	2.1	11.3	10.9	8.1
20	5	5	10	12.8	18.3	26.6	19.2
21	2	5	1	17.0	25.4	28.1	23.5
22	3	6	6	23.4	33.8	37.5	31.6
23	4	11	2	31.9	49.3	40.6	40.6
24	8	7	4	48.9	59.2	46.9	51.7
25	6	4	3	61.7	64.8	51.6	59.4
26	5	6	2	72.3	73.2	54.7	66.8
27	2	6	4	76.6	81.7	60.9	73.1
28	1	3	4	78.7	85.9	67.2	77.3
29	0	2	6	78.7	88.7	76.6	81.3
30	1	4	3	80.9	94.4	81.3	85.5
31	3	1	3	87.2	95.8	85.9	89.6
32	3	2	1	93.6	98.6	87.5	93.2
33	2	1	4	97.9	100	93.4	97.2
34	0	0	3	97.9	100	98.4	98.8
35	1	0	1	100	100	100	100
Total dead	47	71	64				
No. in assay	70	75	70				
% dead	67.1	94.7	91.4				

APPENDIX 5

APPENDIX TABLE 5.1

*Bam*HI restriction fragments of AoGV-E DNA and their secondary *Bgl*II fragments

	1° Enzyme: single digest ***Bam*HI**		2° Enzyme: Double digest ***Bgl*II**	
Band	Fragment	Size(kbp)	Fragments	Summation of fragment sizes (kbp)
1	A	37562	a, e, g, l, m, n, o	37456
2	B	16474	c, j, p	16882
3	C	15052	b, k, r	15385
3	D	14867	d, i, q	15256
4	E	7442	f	7417
4	F	7215	h	7294
5	G	399	s	399
		99011		**100089**

APPENDIX TABLE 5.2

*Bgl*II restriction fragments of AoGV-E DNA and their secondary *Bam*HI fragments

	1° Enzyme: single digest ***Bgl*II**		2° Enzyme: Double digest ***Bam*HI**	
Band	Fragment	Size (kbp)	Fragments	Summation of fragment sizes (kbp)
1	A	28096	c, s, h, d	28339
2	B	13838	a	13523
2	C	13523	q, b	13523
3	D	11345	k, f, o	11681
4	E	9511	e	9511
5	F	7314	g	7317
6	G	4206	i	4243
7	H	4206	j	4243
8	I	3392	l, p	3485
9	J	1846	m	1846
10	K	1678	n	1678
11	L	700	r	700
		99655		**100089**

APPENDIX TABLE 5.3

*Bam*HI restriction fragments of AoGV-E DNA and their secondary *Pst*I fragments.

	1° Enzyme: single digest ***Bam*HI**		2° Enzyme: Double digest ***Pst* I**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	37,562	h, a, d	37,569
2	B	16,474	b	16,474
3	C	15,052	j, i, g, k	15,658
3	D	14,867	c, m	15,062
4	E	7442	e	7520
4	F	7215	f	7314
5	G	399	l	390
		99,011		**99,987**

APPENDIX TABLE 5.4

*Pst*I restriction fragments of AoGV-E DNA and their secondary *Bam*HI fragments.

	1° Enzyme: single digest ***Pst*I**		2° Enzyme: Double digest ***Bam*HI**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	46,521	d, b, l, f, c, m	47,201
2	B	25,884	a	25,884
2	C	13,523	k, e, h	14,114
3	D	6334	g	6334
4	E	3335	i	3335
5	F	3119	j	3119
		98,716		**99,987**

APPENDIX TABLE 5.5

*Bam*HI restriction fragments of AoGV-E DNA and their secondary *Sac*I fragments.

	1° Enzyme: single digest ***Bam*HI**		2° Enzyme: Double digest ***Sac*I**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	37,562	a, e	39,179
2	B	16,474	b	16,474
3	C	15,052	h, d	15,921
3	D	14,867	c	15,052
4	E	7442	f	7387
4	F	7215	i, g	7179
5	G	399	j	399
		99,011		**101,591**

APPENDIX TABLE 5.6

*Sac*I restriction fragments of AoGV-E DNA and their secondary *Bam*HI fragments

	1° Enzyme: single digest ***Sac*I**		2° Enzyme: Double digest ***Bam*HI**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	46,000	d, f, a	46,566
2	B	32,000	e, b, j, i	32,345
2	C	23,000	g, c, h	22,680
		101,000		**101,591**

APPENDIX TABLE 5.7

*Bgl*II restriction fragments of AoGV-E DNA and their secondary *Pst*I fragments.

	1° Enzyme: single digest ***Bgl*II**		2° Enzyme: Double digest ***Pst*I**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	28,096	a	28,096
2	B	13,838	d, g	14,050
3	C	13,523	o, l, k, f,	13,828
3	D	11,345	b	11,563
4	E	9511	p, c	9728
4	F	7314	e	7416
5	G	4206	h	4281
6	H	4206	i	4281
7	I	3392	j	3392
8	J	1846	m	1846
9	K	1678	n	1678
10	L	700	q, r	700
		99,655		**100,859**

APPENDIX TABLE 5.8

*Pst*I restriction fragments of AoGV-E DNA and their secondary *Bgl*II fragments

	1° Enzyme: single digest ***Pst*I**		2° Enzyme: Double digest ***Bgl*II**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	46,521	g, j, i, a, h, o	47,424
2	B	25,884	c, e, m, d	26,592
2	C	13,523	r, b, n, p	14,036
3	D	6334	f, q	6353
4	E	3335	k	3335
5	F	3119	l	3119
		98,716		**100,859**

APPENDIX TABLE 5.9

*Bgl*II restriction fragments of AoGV-E DNA and their secondary *Sac*I fragments

	1° Enzyme: single digest ***Bgl*II**		2° Enzyme: Double digest ***Sac*I**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	28,096	b, a	30,110
2	B	13,838	l, d	14,150
3	C	13,523	k, e	13,958
3	D	11,345	c	11,954
4	E	9511	f	9719
4	F	7314	g	7387
5	G	4206	h	4206
6	H	4206	i	4206
7	I	3392	j	3099
8	J	1846	m	1846
9	K	1678	n	1678
10	L	700	o	700
		99,655		**103,013**

APPENDIX TABLE 5.10

*Sac*I restriction fragments of AoGV-E DNA and their secondary *Bgl*II fragments

	1° Enzyme: single digest ***Sac*I**		2° Enzyme: Double digest ***Bgl*II**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	46,000	e, o, c, n, m, g, f, l	46,530
2	B	32,000	d, j, i, b,	32,534
2	C	23,000	a, h, k	23,949
		101,000		**103,013**

APPENDIX TABLE 5.11

*Pst*I restriction fragments of AoGV-E DNA and their secondary *Sac*I fragments

	1° Enzyme: single digest ***Pst*I**		2° Enzyme: Double digest ***Sac*I**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	46,521	a, b	47,702
2	B	25,884	c, f	27,719
3	C	13,523	d	13,523
3	D	6335	e	6334
4	E	3335	g	3335
4	F	3119	i, h	3233
		98,716		**101,846**

APPENDIX TABLE 5.12

*Sac*I restriction fragments of AoGV-E DNA and their secondary *Pst*I fragments

	1° Enzyme: single digest ***Sac*I**		2° Enzyme: Double digest ***Pst*I**	
Band	Fragment	Size (bp)	Fragments	Summation of fragment sizes (bp)
1	A	46,000	h, g, e, d, c	46,662
2	B	32,000	f, a	31,785
2	C	23,000	b, i	23,399
		101,000		**101,846**

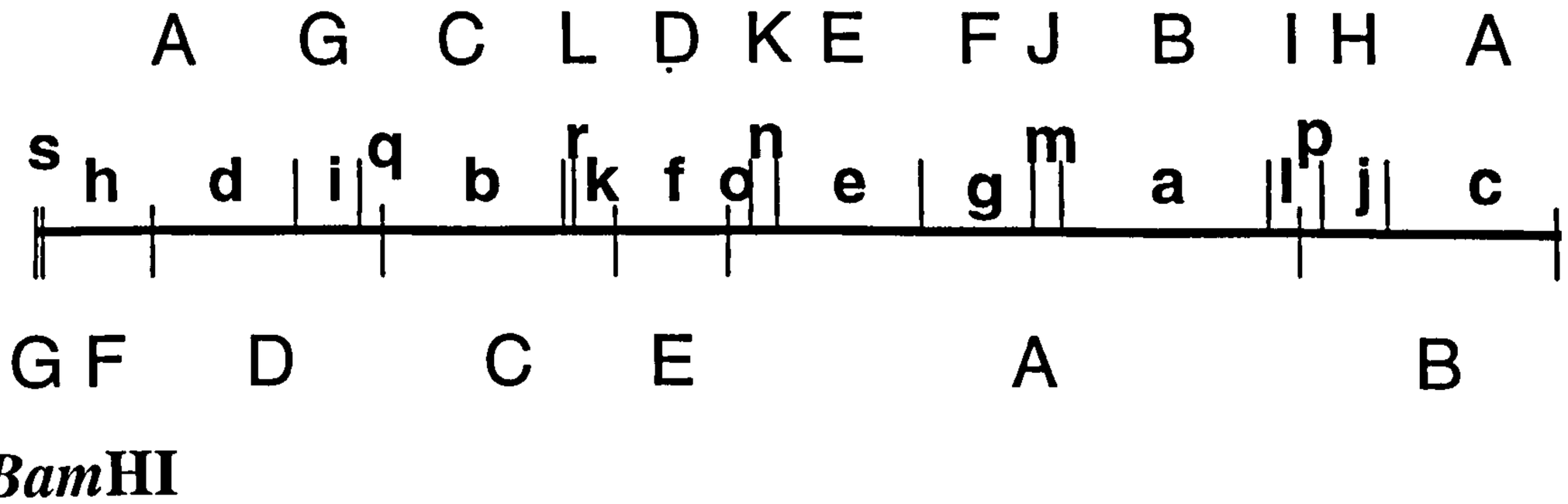
APPENDIX 6

Restriction maps of AoGV-E. The upper case letters denote single digest fragments which are above and below the maps and lower case letters denote the double digest fragments of the two enzymes.

A= *Bgl*II and *Bam*HI
B= *Bam*HI and *Pst*I
C= *Bam*HI and *Sac*I
D= *Bgl*II and *Pst*I
E= *Bgl*II and *Sac*I
F= *Pst*I and *Sac*I

A

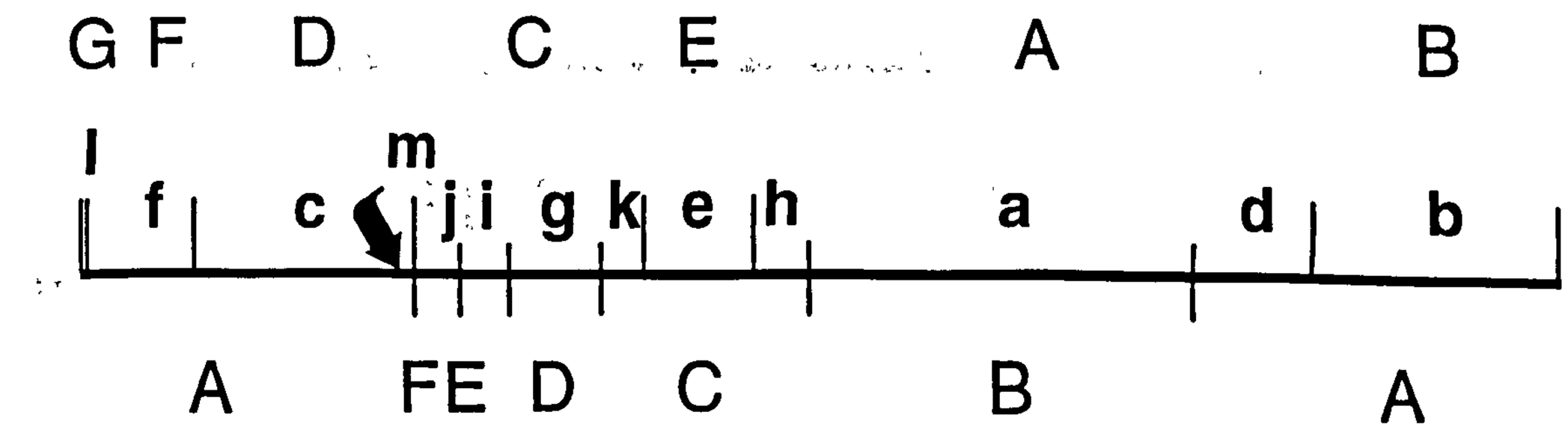
***Bgl*II**



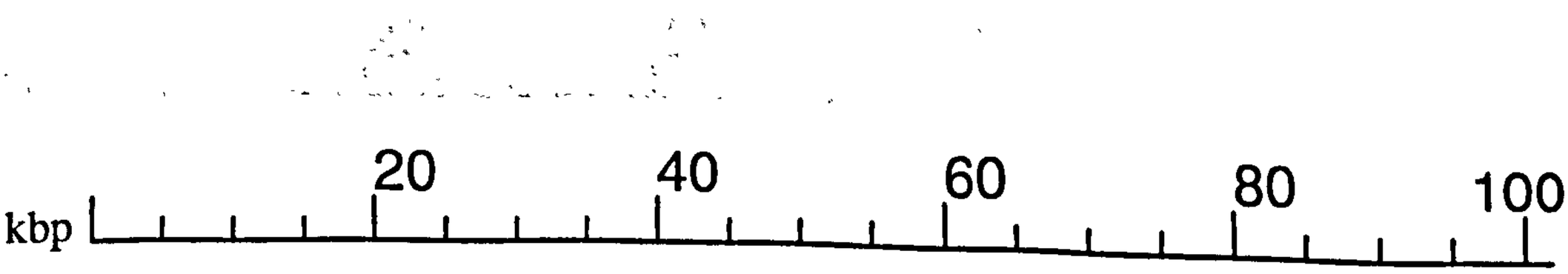
***Bam*HI**

B

***Bam*HI**

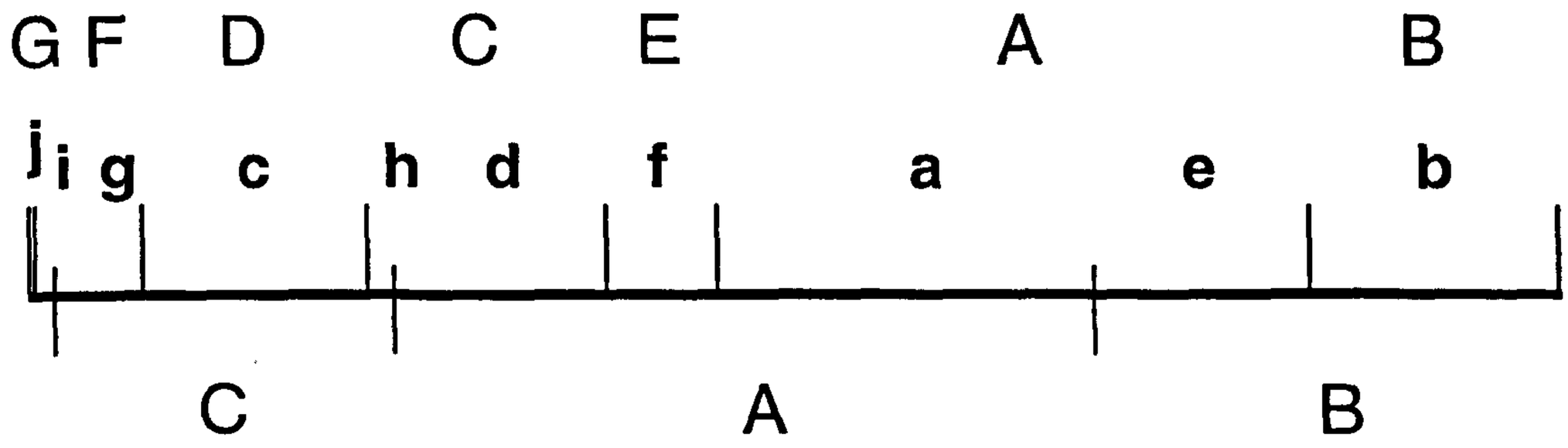


***Pst*I**



C

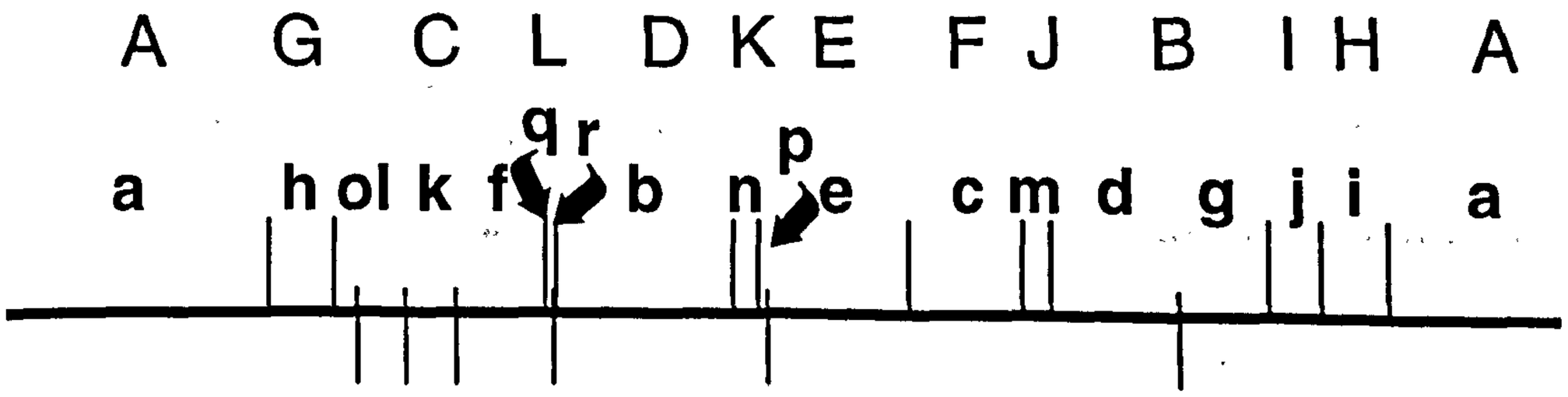
***Bam*HI**



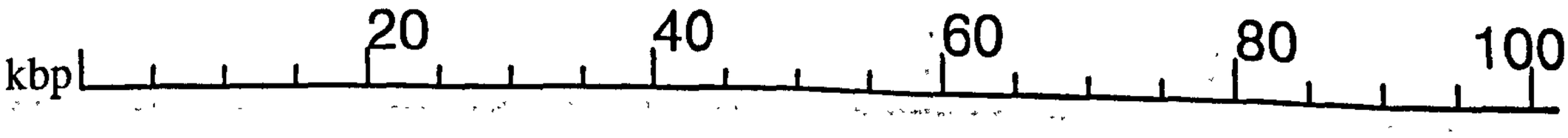
***Sac*I**

D

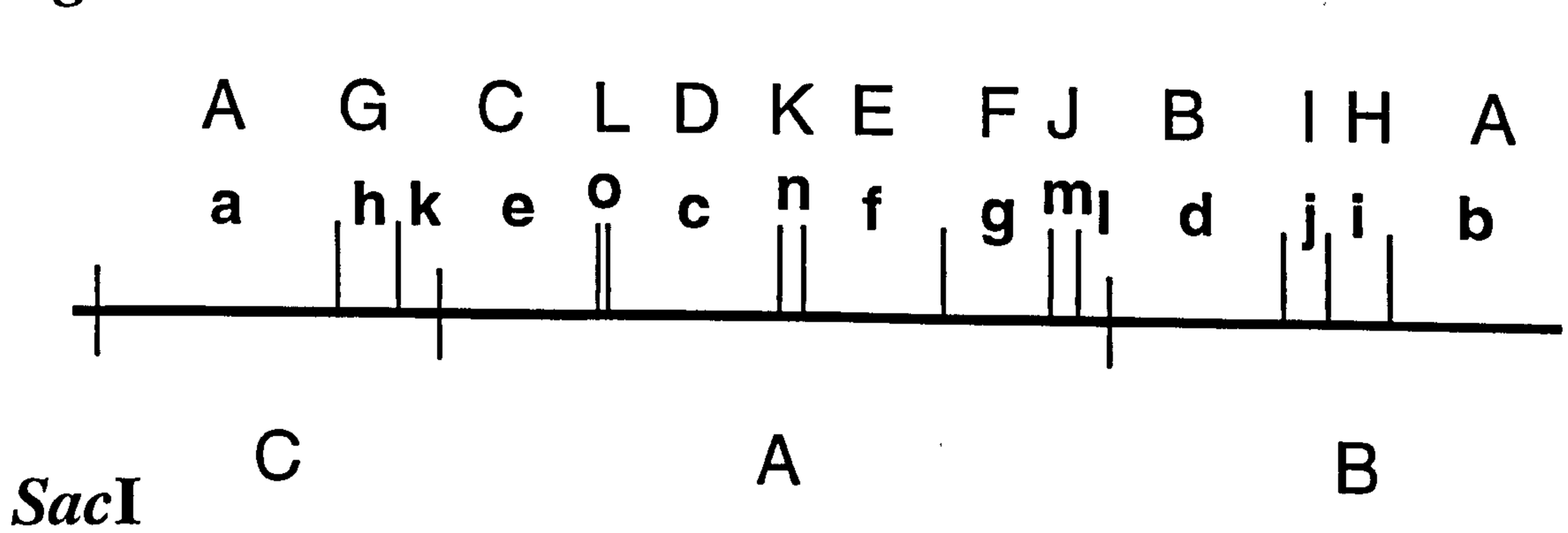
***Bgl*II**



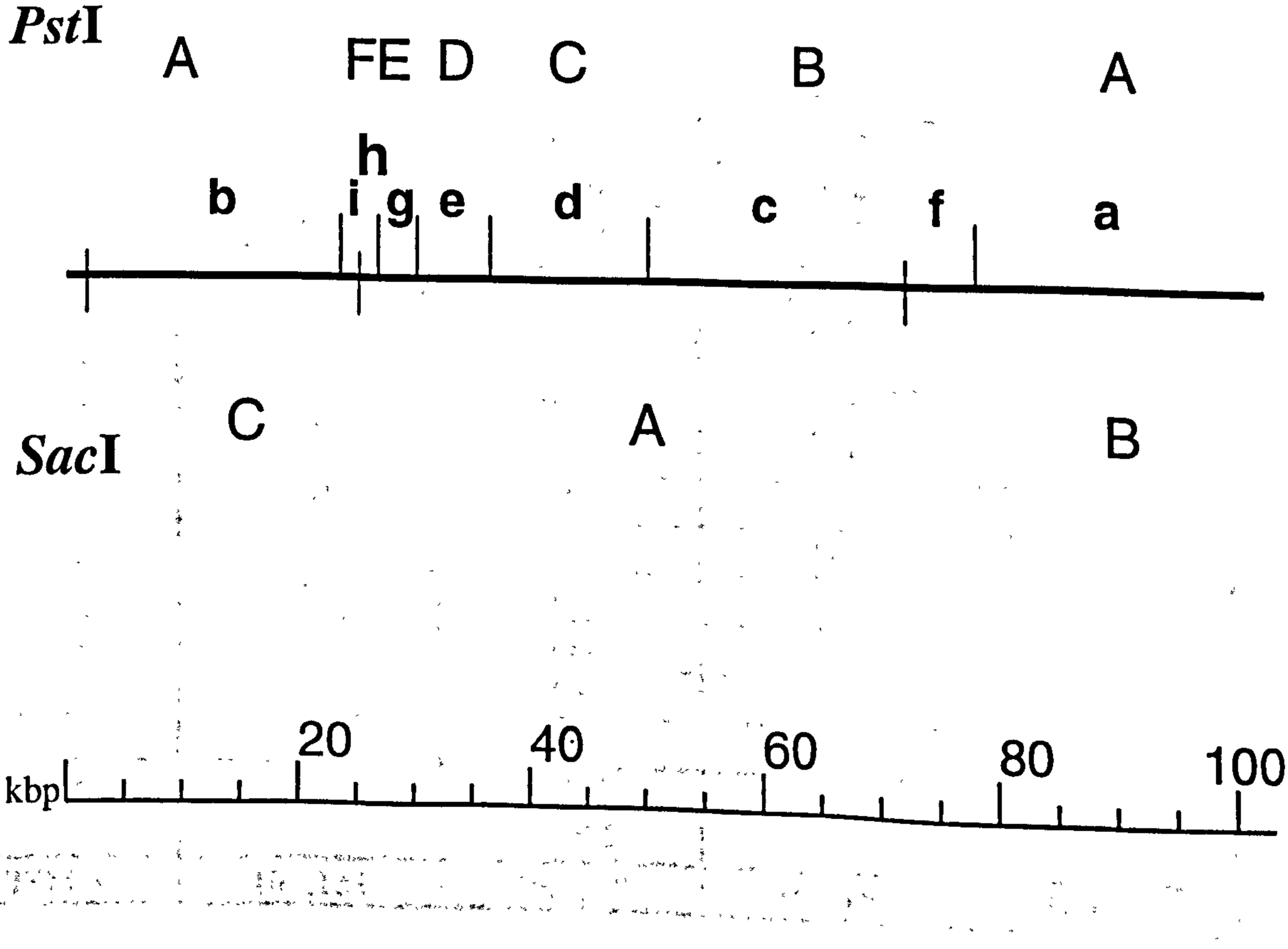
***Pst*I**



E


BglII
A G C L D K E F J B I H A
a h k e o c n f g m l d j i b
C A B
SacI

F


PstI
A FE D C B A
b i h g e d c f a
C A B
SacI
kbp
20 40 60 80 100

APPENDIX 7

APPENDIX TABLE 7.1

Estimated sizes in bp of restriction fragments of AoGV-E DNA digested with *Eco*RI and one other mapped restriction enzyme.

Fragment	*Eco*RI + *Bam*HI	*Eco*RI + *Bgl*II	*Eco*RI + *Pst*I	*Eco*RI + *Sac*I
a	14,593	13,152	12,364	12,794
b	12,478	10,999	12,364	12,364
c	10,999	10,095	10,999	12,364
d	7417	8972	10,095	10,999
e	6722	5013	6334	10,095
f	6218	4800	6218	6218
g	5771	4206	5013	5013
h	5013	3990	3990	3639
i	3990	3392	3335	3312
j	3639	3392	3119	2878
k	2878	3054	2878	2691
l	2691	2878	2691	2691
m	2338	2691	2338	2074
n	2074	2338	2074	2050
o	1771	2206	2050	1771
p	1660	2074	2050	1500
q	1322	2050	1771	1332
r	1322	1846	1771	1332
s	1322	1771	1636	1221
t	1221	1678	1332	904
u	967	1660	1332	838
v	967	1496	1221	718
w	904	1332	1150	678
x	718	1332	718	577
y	577	1318	587	444
z	444	904	577	390
α	390	850	444	
β	361	718	390	
χ	210	700	321	
δ	154	577		
ε		444		
ϕ		390		
γ		371		
η		< 100		
ι		< 100		
TOTAL	**101,131**	**102,689**	**101,162**	**100,887**

APPENDIX TABLE 7.2

Re-calculated sizes in bp of single digest fragments of *Bam*HI, *Bgl*II, *Pst*I and *Sac*I calculated from the summation of smaller double digest fragments. Also estimated sizes in bp of *Eco*RI fragments.

	***Bam*HI**	***Bgl*II**	***Pst*I**	***Sac*I**	***Eco*RI**
A	38,096	28,187	47,448	46,007	15,485
B	16,845	13,949	26,389	32,219	12,364
C	15,794	13,932	14,179	22,711	12,364
D	15,219	11,706	6353		10,999
E	7442	9728	3335		10,095
F	7179	7314	3233		6218
G	362	4206			5013
H		4206			3990
I		3485			3639
J		1846			2878
K		1678			2691
L		700			2338
M					2074
N					2050
O					1771
P					1332
Q					1332
R					1221
S					904
T					718
U					577
V					444
W					390
TOTAL	**100,937**	**100,937**	**100,937**	**100,937**	**100,887**

APPENDIX 8

Nucleotide sequence of 4460 bp in the granulin region of the AoGV-E1 genome. Deduced amino acid sequences of putative genes obtained by ORF searches are shown with single-letter amino acid codes under the nucleotide sequence. ORF names are shown at the start of the amino acid sequence. The stop codons are indicated by an asterisk (*).

```
TAAATATAAATTGTAATATTAGTTATTTTTTTTGTGTGATTCTGTATATAACTAATGATATTATTGACTGAATATTGGTTATAAAATAAAACGATATAAT      100

AAAATTTGTTTATTTATTTGAACAACCTAATCACTTTATTGGTATACCATACAGATTTGTGTTGCGGTGTTGACAAGTCGTCGTTAATACGTTTTTTGTA      200
            *  K  F  L  R  I  V  K  N  T  Y  W  V  S  K  H  Q  P  T  S  L  D  D  N  I  R  K  K  Y

TTGTACAATGTTACTCTTGTAGTTATCATAGTTTTGCATCATCAAATCAATGGTCTGATTTAGATTGGATATTGATCTGGCGCGTAAAGTTTCTCCAATG      300
 Q  V  I  N  S  K  Y  N  D  Y  N  Q  M  M  L  D  I  T  Q  N  L  N  S  I  S  R  A  R  L  T  E  G  I

TTCAATTGTTTGATGTTGCGAGCGTTTAAAAATTGATCACCCATCAAAGGCACAACAATCATAGGTATTTCATTATCTACCGCTTCGTCTATCGACAAAA      400
N  L  Q  K  I  N  R  A  N  L  F  Q  D  G  M  L  P  V  V  I  M  P  I  E  N  D  V  A  E  D  I  S  L  I

TGCCTCCTTGTGTTATGAACAGCTTGACACGAGGGTTCTTCAAAATATTCCTCTGCGGTAACCATTTTCTGACGAGCACATTGTTGGTGTTGTTGTAGTC      500
  G  G  Q  T  I  F  L  K  V  R  P  N  K  L  I  N  R  Q  P  L  W  K  R  V  L  V  N  N  T  N  N  Y  D

ATGTTCCGGAGTGGCGTGTAATAAAATTTTGAAAGGCGAAGCGGCCAGTGCGACGAATACGTTTTTCAATATGGTGTTGTTGAAATTGTTTGATCCGAAA      600
 H  E  P  T  A  H  L  L  I  K  F  P  S  A  A  L  A  V  F  V  N  K  L  I  T  N  N  F  N  N  S  G  F

CTGACATATATTATTGTTGTGTTGGGAGCTGCCAAAAAATTGGTTAGTTTCACATCGACATCGCTCAATATAGGCGGCGGTGTTGTCAAATGAAGACCGC      700
S  V  Y  I  I  T  T  N  P  A  A  L  F  N  T  L  K  V  D  V  D  S  L  I  P  P  P  T  T  L  H  L  G  G

CCAAATATTGTACCTTAGAATTGATGGGTCTGTCGTTATCAAAATGGGACGCGGTATTGATAAACAACAATTTGACTCTCTTCTTCAATTCATCTATTGC      800
  L  Y  Q  V  K  S  N  I  P  R  D  N  D  F  H  S  A  T  N  I  F  L  L  K  V  R  K  K  L  E  D  I  A

GGGTATGTCTTGACCAAAATGTTTTGTGAGTAAATCGTGTTGTATGACGTCAAATTTATTCCATTCTTCATTGATTCGTAATTCCTCGTCGTCGTGACAT      900
 P  I  D  Q  G  F  H  K  T  L  L  D  H  Q  I  V  D  F  K  N  W  E  E  N  I  R  L  E  E  D  D  H  C

TTTTCTTCATTTTCACAATTCAATGTGTTAAATACATTGGGATATATTTGGTTGTTATAGCTTGCGGTTTTGTTTAATAGGGTGTTATAATTGTCGTTGG     1000
K  E  E  N  E  C  N  L  T  N  F  V  N  P  Y  I  Q  N  N  Y  S  A  T  K  N  L  L  T  N  Y  N  D  N  T

TGGCTCGTCCCGAAGAGATCCAAATTATGGGCGATTTGTACACATGTCCAAAGTATAGGTTAATGTTCAGCATGGACTCGCATACTATCAAATCAAATTT     1100
  A  R  G  S  S  I  W  I  I  P  S  K  Y  V  H  G  F  Y  L  N  I  N  L  M  S  E  C  V  I  L  D  F  K

GACATTTGTGTTGTTTAATAACGCAACCACACCATCGCTGTTAAATTGATCGACGACCATATTTAACAAATCACGGTATTGTTCTGCAACAAAAATTTGA     1200
 V  N  T  N  N  L  L  A  V  V  G  D  S  N  F  Q  D  V  V  M  N  L  L  D  R  Y  Q  E  A  V  F  I  Q

TCAGCTTTGGCTGTGCTCATTAAAGCGCTGTACACGTTCAAGGTGTTCTTTGATGTGTCGATTTCTGTTAAATGTTTGACATGTCTAGGCATGGGAGTTA     1300
D  A  K  A  T  S  M  L  A  S  Y  V  N  L  T  N  K  S  T  D  I  E  T  L  H  K  V  H  R  P  M  P  T  I

TGACGGTAACATTATGTCCGTTTTTAACCAAAGAGTCCGTATAAGCAGAATAAACCAAATGATGACTATATGACGGTATTGGAAAAACGCATAATATTCT     1400
  V  T  V  N  H  G  N  K  V  L  S  D  T  Y  A  S  Y  V  L  H  H  S  Y  S  P  I  P  F  V  C  L  I  R

AGCGGACGAGACAACGGTAAAAAGTAACGACAACACAATCAACGAATACATGTCTAATATGCTACTTATTTTTGCATTAAATGCACTCTATTTATACCAC     1500
 A  S  S  V  V  T  F  L  L  S  L  V  I  L  S  Y  M  < start ogt

ATCTTAATGTGTGTGTGCGTATAATGCAAGACGTTCGTGTGCAATTTTTGTGTAAAGAGAAAAAAGAGGTGATAACCTTCTTATCAGTTCTATTGAAAAA     1600
       start ORF 99R >  M  Q  D  V  R  V  Q  F  L  C  K  E  K  K  E  V  I  T  F  L  S  V  L  L  K  N

CATTTTGAACGGTATCGAGTCGATAAATTGTCATAATTGCAACGCGTCATTCAAAAGGTTAAAAGAAAGTCAATACATATTTATATTTGCGATAGTAAAC     1700
  I  L  N  G  I  E  S  I  N  C  H  N  C  N  A  S  F  K  R  L  K  E  S  Q  Y  I  F  I  F  A  I  V  N

GATTATAACGACACAACAGATGACGTGTTGAAATTTTGTTGTTTAAAATGTTGTAAAATTGAAAAAAATAATTTAATGGAAGTCATAGAGTTATATCCAA     1800
 D  Y  N  D  T  T  D  D  V  L  K  F  C  C  L  K  C  C  K  I  E  K  N  N  L  M  E  V  I  E  L  Y  P

CTTTGACCTTGCAAAACGTAAATAAGCTAATGTATTATGGAATGTTAAAAAAGTTCATGTTTGATTTTCACGACAGCGATCAAGTTTCTTATAAAAAATA     1900
T  L  T  L  Q  N  V  N  K  L  M  Y  Y  G  M  L  K  K  F  M  F  D  F  H  D  S  D  Q  V  S  Y  K  K  Y

TGTAATATCGTCCAGCCTTAGCGCTGTGCTAGATCAAATGTTTTTAGAAAAACAAAATAATGAAGAAATAATCAGACTTAGTTTGAGACGCAATAATCAA     2000
  V  I  S  S  S  L  S  A  V  L  D  Q  M  F  L  E  K  Q  N  N  E  E  I  I  R  L  S  L  R  R  N  N  Q

ATAGTGGCTGAAGATTTTTTGCACGATTTACGCGTAGACTATTGCGACAAATATAATTTTGAAAAAGATTTGTCTATAAATAAAGAATTCGTGGAAGCGG     2100
 I  V  A  E  D  F  L  H  D  L  R  V  D  Y  C  D  K  Y  N  F  E  K  D  L  S  I  N  K  E  F  V  E  A

TAGACGCTCACTGCAATTTAAACGTGTATCATTTGGATGTATACTATAAAACTTACAATTTATACAGTCCTTTTGTTGTACAATTTAACAGAAACAATTT     2200
V  D  A  H  C  N  L  N  V  Y  H  L  D  V  Y  Y  K  T  Y  N  L  Y  S  P  F  V  V  Q  F  N  R  N  N  L
```

```
AAACGAATGCGCCTATTGTGTTAATAAAATTAGCAAAGAAACTAATCACCCTATATTGCATTGTAGCTTGTGCGGCGCGACGGATCCTAATTATTTTATT        2300
  N  E  C  A  Y  C  V  N  K  I  S  K  E  T  N  H  P  I  L  H  C  S  L  C  G  A  T  D  P  N  Y  F  I

AGAAACAGACGTATGATGATACCGTTTTGGAGAGCCGACTACAATTATAATTTAGTATATTGGAGGTTGATAAAAAACAAAAATTTACCTTGCAGTTTAA        2400
  R  N  R  R  M  M  I  P  F  W  R  A  D  Y  N  Y  N  L  V  Y  W  R  L  I  K  N  K  N  L  P  C  S  L

TGATGTACGCTGTCGATGTTAAAGTCGATGTTTAATTATAAGGAATTTTAATTTTATCAAAACTATGGGATATAACAAATCTTTGCGTTACAGTCGTCAC        2500
  M  M  Y  A  V  D  V  K  V  D  V  *                    start granulin >  M  G  Y  N  K  S  L  R  Y  S  R  H

GAAGGCACCACCTGTGTTATCGACAATCACCACCTTAAAAGTCTTGGTTCGGTGTTGAACGATGTGCGTCGTAAAAAAGACCGAATCCGCGAGGCGGAAT        2600
  E  G  T  T  C  V  I  D  N  H  H  L  K  S  L  G  S  V  L  N  D  V  R  R  K  K  D  R  I  R  E  A  E

ACGAACCTATTCTAGACATTGCCGACCAGTACATGGTTACGGAGGATCCTTTTCGTGGTCCGGGTAAAAATGTACGCATCACCTTGTTCAAAGAAATTCG        2700
  Y  E  P  I  L  D  I  A  D  Q  Y  M  V  T  E  D  P  F  R  G  P  G  K  N  V  R  I  T  L  F  K  E  I  R

TCGAGTACATCCCGATACGATGAAGCTTGTGTGCAACTGGAGCGGGAAAGAGTTTCTGAGAGAGACTTGGACTCGTTTCATTTCCGAAGAATTCCCGATC        2800
  R  V  H  P  D  T  M  K  L  V  C  N  W  S  G  K  E  F  L  R  E  T  W  T  R  F  I  S  E  E  F  P  I

ACCACCGATCAGGAGATCATGGACCTGTGGTTCGAGATTCAGCTGCGACCGATGCATCCCAATAGGTGTTACAAGTTCACGATGCAATACGCGCTGGGCG        2900
  T  T  D  Q  E  I  M  D  L  W  F  E  I  Q  L  R  P  M  H  P  N  R  C  Y  K  F  T  M  Q  Y  A  L  G

CTCACCCCGATTACGTGGCTCACGATGTTATCCGTCAACACGATCCTTACTACGTAGGACCCAACAACATTGAACGTATCAATCTGAGCAAGAAAGGTTT        3000
  A  H  P  D  Y  V  A  H  D  V  I  R  Q  H  D  P  Y  Y  V  G  P  N  N  I  E  R  I  N  L  S  K  K  G  F

CGCTTTCCCACTGACTTGTCTCCAATCAGTTTACAATGCCACCTTTGAAAGGTTCTTTGATGATGTGCTGTGGCCCTATTTCCATCGCCCACTAGTCTAT        3100
  A  F  P  L  T  C  L  Q  S  V  Y  N  A  T  F  E  R  F  F  D  D  V  L  W  P  Y  F  H  R  P  L  V  Y

ATCGGTACCACGTCTGCCGAAGTCGAAGAGATTATGATTGAGGTGTCGCTTCTGTTTAAGATCAAAGAATTCGCACCCGACGTGCCGCTGTTCACGGGAC        3200
  I  G  T  T  S  A  E  V  E  E  I  M  I  E  V  S  L  L  F  K  I  K  E  F  A  P  D  V  P  L  F  T  G

CGGCGTATTAAGCAAAGCTTTCTGATTTGTCACCCGTTTCTATGTCAATAAGGTTGATTTCTTGTTGTTCATTAATAACCGGCGGTGATGGTGTTGTTTT        3300
  P  A  Y  *
     *  A  F  S  E  S  K  S  D  G  T  E  I  D  I  L  N  I  E  Q  Q  E  N  I  V  P  P  S  P  T  T  K

ATCTATTTTTGTTGTAGTTGTTAGACGTGGTGATTTAATTATCATATAATATAGTGTATCTAAAAATTCTACTACTTCGTCCAGATTCATACCGACCAGG        3400
  D  I  K  T  T  T  T  L  R  P  S  K  I  I  M  Y  Y  L  T  D  L  F  E  V  V  E  D  L  N  M  G  V  L

TCTGTGTTATCGTTGTTTTGTAACGCGGATATTTTTCTTTTAAGACTCGCATTCCATTTCATTTTTGGTATCGCATCTCGAGCGTCGACTTTGAAATTAG        3500
  D  T  N  D  N  N  Q  L  A  S  I  K  R  K  L  S  A  N  W  K  M  K  P  I  A  D  R  A  D  V  K  F  N  S
                                                                                    start protein

AGTTCCTCACGATGTCCAACAAATCCATATCAAGAGTGGTGCAAGATTTGAAAAATATTCAAATAGTTAAAAAATTATGCGACAACAGCAGCAACAGCGG        3600
  N  R  V  I  D  L  L  D  M  < start ORF 1L
   kinase >  M  S  N  K  S  I  S  R  V  V  Q  D  L  K  N  I  Q  I  V  K  K  L  C  D  N  S  S  N  S  G

TTCTTATGAAAACTTATATGTTTGTAAAAAAAAAGGCGATCCCAATCAATACGTTTGTAAAATAATCAAAGAGAAAATGTTTAACCCATTAGAGTTTGCG        3700
  S  Y  E  N  L  Y  V  C  K  K  K  G  D  P  N  Q  Y  V  C  K  I  I  K  E  K  M  F  N  P  L  E  F  A

ATCGCGAAACTTATGAGCAACAACGTTAATTTCATCGACGTGTATAATTGTTATTATACAAAAAAAGGTCACGTGATTCTCATAATGGATTACGTTGTCG        3800
  I  A  K  L  M  S  N  N  V  N  F  I  D  V  Y  N  C  Y  Y  T  K  K  G  H  V  I  L  I  M  D  Y  V  V

ATGGAGATTTGTTTGAATTGGTAAAAAGCAAGCAGAGCTCAATATTAGACGAGGCGTTGTGTCGTAAGATATTGATAAATTTGATTACCGCTCTAAACGA        3900
  D  G  D  L  F  E  L  V  K  S  K  Q  S  S  I  L  D  E  A  L  C  R  K  I  L  I  N  L  I  T  A  L  N  D

TCTGCACGCCCAGCAATTTATTCACAACGATGTGAAATTGGAAAACTTGCTATTCGACATAAAACGCAAACGTTTGTATTTGTGCGATTTTGGATTGGTA        4000
  L  H  A  Q  Q  F  I  H  N  D  V  K  L  E  N  L  L  F  D  I  K  R  K  R  L  Y  L  C  D  F  G  L  V

AAATGTATTAACGTTCCGTCACATTACGACGGGACCACTATTTATTTTTCGCCTGAAAAAATTGCAAAAATTCCATGCACGCAATCGTTCGACTGGTGGG        4100
  K  C  I  N  V  P  S  H  Y  D  G  T  T  I  Y  F  S  P  E  K  I  A  K  I  P  C  T  Q  S  F  D  W  W

CGGTAGGAATAGTTGCCTACGAATTGTTATCAAAAAACTATCCATTCGAGTTCGACGAAGAAGAAGAAGACGAAATAAATCCCAAAGAAATGATGAACAT        4200
  A  V  G  I  V  A  Y  E  L  L  S  K  N  Y  P  F  E  F  D  E  E  E  E  D  E  I  N  P  K  E  M  M  N  I

ATACAAGCAGCCTTTAAAAAAGATTCCTAACATATCTAAGAACGCCATGGATTTTGTTAGACAAATGCTAATGTTAGACATTAACAAACGTCTGAGTACG        4300
  Y  K  Q  P  L  K  K  I  P  N  I  S  K  N  A  M  D  F  V  R  Q  M  L  M  L  D  I  N  K  R  L  S  T

TACGATCAAATAATAAAACATCCATTTTTGAATATTTAATCAAAGTGTTTTATTTGGTGTAAAATCAAAGCGGTTGCATTGTCGTAGCATTCTATAGCTT        4400
  Y  D  Q  I  I  K  H  P  F  L  N  I  *

TGTACCGCGTCAACAAGCATTGTGCCATTTTAAATTCATCAGCATGCGCCATGTACAGTT        4460
```

APPENDIX 9

APPENDIX TABLE 9.1

Cumulative number of dead larvae, percentage mortality of larvae infected in assay and percentage mortality of larvae that died at each time point (h p.i.), for genotypes CpGV-M1 and CpGV-R1 in bioassay 1.

M1				R1			
h p.i.	Cum. dead	% dead	% infected dead	h p.i.	Cum. dead	% dead	% infected dead
95	1	2.17	2.56	95	0	0	0
100.5	4	8.70	10.26	100.5	1	2.27	2.70
107	9	19.57	23.08	107	1	2.27	2.70
114	17	36.96	43.59	114	2	4.55	5.41
119	23	50.00	58.97	119	17	38.64	45.95
125	31	67.39	79.49	125	22	50.00	59.46
131	37	80.43	94.87	131	32	72.73	86.49
140	38	82.61	97.44	140	35	79.55	94.59
148	39	**84.78**	100	148	37	**84.09**	100
No. in bioassay	46			44			

APPENDIX TABLE 9.2

Cumulative number of dead larvae, percentage mortality of larvae infected in assay and percentage mortality of larvae that died at each time point (h p.i.), for genotypes CpGV-R3 and CpGV-R3^{R} in bioassay 1.

R3				R3^{R}			
h p.i.	Cum. Dead	% dead	% infected dead	h p.i.	Cum. dead	% dead	% infected dead
95	0	0	0	95	0	0	0
100.5	0	0	0	100.5	0	0	0
107	0	0	0	107	1	2.13	2.78
114	8	17.39	24.24	114	5	10.64	13.89
119	14	30.43	42.42	119	11	23.40	30.56
125	20	43.48	60.61	125	21	44.68	58.33
131	29	63.04	87.88	131	31	65.96	86.11
140	30	65.22	90.91	140	35	74.47	97.22
148	31	67.39	93.94	148	35	74.47	97.22
171	32	69.57	96.97	171	35	74.47	97.22
177	33	**71.74**	100	177	36	**76.60**	100
No. in bioassay	46			47			

APPENDIX TABLE 9.3

Cumulative number of dead larvae, percentage mortality of larvae infected in assay and percentage mortality of larvae that died at each time point (h p.i.), for genotypes CpGV-M1 and CpGV-R1 in bioassay 2.

M1				R1			
h p.i.	Cum. dead	% dead	% infected dead	h p.i.	Cum. dead	% dead	% infected dead
96	1	2.17	2.78	96	0	0	0
101.5	3	6.52	8.33	101.5	1	2.33	2.70
107.5	8	17.39	22.22	107.5	4	9.30	10.81
114.5	12	26.09	33.33	114.5	5	11.63	13.51
119.5	18	39.13	50.00	119.5	9	20.93	24.32
125.5	26	56.52	72.22	125.5	19	44.19	51.35
131.5	33	71.74	91.67	131.5	24	55.81	64.86
138.5	34	73.91	94.44	138.5	32	74.42	86.49
143.5	35	76.09	97.22	143.5	33	76.74	89.19
149.5	35	76.09	97.22	149.5	35	81.40	94.59
162	35	76.09	97.22	162	37	86.05	100.00
170.5	36	**78.26**	100.00	170.5	37	**86.05**	100.00
No. in bioassay	46			43			

APPENDIX TABLE 9.4

Cumulative number of dead larvae, percentage mortality of larvae infected in assay and percentage mortality of larvae that died at each time point (h p.i.), for genotypes CpGV-R3 and CpGV-R3^{R} in bioassay 2.

R3				R3^{R}			
h p.i.	Cum. Dead	% dead	% . infected dead	h p.i.	Cum. dead	% dead	% infected dead
96	0	0	0.00	96	0	0	0
101.5	1	2.08	3.13	101.5	2	4.55	5.88
107.5	2	4.17	6.25	107.5	3	6.82	8.82
114.5	5	10.42	15.63	114.5	5	11.36	14.71
119.5	5	10.42	15.63	119.5	9	20.45	26.47
125.5	14	29.17	43.75	125.5	14	31.82	41.18
131.5	18	37.50	56.25	131.5	25	56.82	73.53
138.5	24	50.00	75.00	138.5	30	68.18	88.24
143.5	26	54.17	81.25	143.5	32	72.72	94.12
149.5	27	56.25	84.38	149.5	33	75.00	97.06
162	31	64.58	96.88	162	33	75.00	97.06
170.5	32	**66.67**	100.00	170.5	34	**77.27**	100
No. in bioassay	48			44			

APPENDIX TABLE 9.5

Cumulative number of dead larvae, percentage mortality of larvae infected in assay and percentage mortality of larvae that died at each time point (h p.i.), for genotypes CpGV-M1 and CpGV-R1 in bioassay 3.

M1				R1			
h p.i.	Cum. dead	% dead	% infected dead	h p.i.	Cum. dead	% dead	% infected dead
102	1	2.17	2.50	102	0	0	0
108	2	4.35	5.00	108	0	0	0
115	9	19.57	22.50	115	1	2.33	3.85
120	19	41.30	47.50	120	3	6.98	11.54
126	20	43.48	50.00	126	8	18.60	30.77
132	26	56.52	65.00	132	13	30.23	50.00
139	37	80.43	92.50	139	18	41.86	69.23
144	37	80.43	92.50	144	23	53.49	88.46
150	38	82.61	95.00	150	23	53.49	88.46
165	40	86.96	100.00	165	25	58.14	96.15
170	40	**86.96**	100.00	170	26	**60.47**	100
No. in bioassay	46			43			

APPENDIX TABLE 9.6

Cumulative number of dead larvae, percentage mortality of larvae infected in assay and percentage mortality of larvae that died at each time point (h p.i.), for genotypes CpGV-R3 and CpGV-R3^{R} in bioassay 3.

R3				R3^{R}			
h p.i	Cum. Dead	% dead	% infected dead	h p. i	Cum. Dead	% dead	% infected dead
102	0	0	0	102	0	0	0
108	1	2.22	2.50	108	0	0	0
115	5	11.11	12.50	115	4	9.30	11.43
120	7	15.56	17.50	120	8	18.60	22.86
126	14	31.11	35.00	126	12	27.91	34.29
132	17	37.78	42.50	132	15	34.88	42.86
139	29	64.44	72.50	139	26	60.47	74.29
144	34	75.56	85.00	144	29	67.44	82.86
150	38	84.44	95.00	150	29	67.44	82.86
165	40	88.89	100	165	33	76.74	94.29
170	40	**88.89**	100	170	35	**81.40**	100
No. in bioassay	45			43			

APPENDIX 10

Alignment of nucleotide sequences of 4332 bp within the *Sal*I-F region of CpGV-M1 and CpGV-R3[R] and the equivalent region in CpGV-R1 and CpGV-R3. The sequences of CpGV-M1 and CpGV-R3[R] were identical in this region and are represented by the upper sequence. The sequences of CpGV-R1 and CpGV-R3 were also identical in this region and are represented by the lower sequence. ORF names and directions (<>) are shown at the start of the sequence. The end of the sequence is denoted by an asterisk(*). Differences between sequences are unshaded. ORF un = no homologue.

```
              10        20        30        40        50
1    TGAATAATGTCCAGTGAGGTGGGAGTAGCCATGCTTAAATAAACAAATTG
1    TGAATAATGTCCAATGAAGTGGGAGTAGCCATGCTTAATTAAACAAATTG

                                      <p74
              60        70        80        90        100
51   GGTGGTA-CAGTATAAATAGTCATTTATATATTTT-----------AACC
51   GGTAGTAACAGTATTAGTAGTCATTTATATATATATTTCTTTTATATTAACC

              110       120       130       140       150
89   TTTAAAAAATACATACTATACAAA---TACTA------CACACACCAAAC
101  TTTAAAAAATACATACTATACAAACTTTACTATTAACACACACACCAAAC

              160       170       180       190       200
130  AAAATAATTCCAACACACAACTCATC-CCATTACAAAACCAAAACAACAC
151  AAAATAATGCCGACACACAACTCATCACCACTACAAGACCAAAGCAACAC

              210       220       230       240       250
179  ATAACACCAATTTTGAACAAATTCTCCACACTCTGAGAAGTCGTCCGCGA
201  ACAACACTAATTTTGAACAA-TTCTCCACACTCTGAGAAGTCGTCCACGA

              260       270  * ORF palindrome   290       300
229  TACATTTTACACCGAAGCCGGTTCAGACAGACGAGTCTGCTTTTTTTCCA
250  TACATTTTACACCGAAGCCGGTTCAGAGAGACGAGTCTGCTTTTTT-CAA

              310       320       330       340       350
279  GAAATTTTAGCTAAAAACTTTTTCTCGGCAAAATCTTGGCGCAAAA-CCA
299  GAAATTTTAGCTAAAAACTTTTTCTCGGCAAAATCTCGGCGCAAAAACCA

              360       370       380       390       400
328  GACTCGTGTCTTTATA-ACCAAACTTGTGGATGGCACCAAAATTGTGAAC
349  GACTCGTGTCTGTATACACCAAACTTGTGCATGGCACCAAAATTATAAAC

              410       420       430       440       450
377  AATGTACACCAAAGTCATGAATCGTTTGCACAGAATTAGTGTACAAAGAA
399  AATGTACACCAAAATCGTGAATCGTTTACACA-----------CAAAGAA

         * ORF repeats  470       480   < ORF palindrome 500
427  GTTGATCACTAGTGCTCTTCTTTGTCGTCGTTAGCATGTTCTACAACCAT
438  GTTGACTACTAGTGCTCTTCTTTGTCGTCGTTAGCATGTTCTACAACCAC

              510       520       530       540       550
477  TTCTGGATGGTGGTCGGCTACAGTCGATTTGTGTGGTTCATCCTTGTGTG
488  TTCTGGATGGTGGTCGGCTACAGTCGATTTGTGTGGTTCATCC-------

              560       570       580       590       600
527  GTTCATCCTTAACTTTGTGTGGTTCATCAACTCTGGGTTCATCAACTCTG
531  --------TCAACTTTGTGTGGTTCTTCGACTCTAAG------------A

              610       620       630       640       650
577  GGTTCATCAACTCTGGGTTCATCAACTCTGGGTTCATCAACTCTGGGTTC
561  GGTTCTTCGACTCTAAG-------A-------------------------

              660       670       680       690       700
627  ATCAACTCTGGGTTCCTCCTGAACTTTGTGTGGTTCTTCAACTCTGGGTT
579  -------------------------------GGTTCTTCGACTCTGGGTT
```

```
                710        720        730        740        750
677 CCTCCTGAACTTTGTGCGGTTCCTCCTCAATTTTAGATTTGTGTTCGTCA
598 CCT-------------------------CCTCAATTATTGATTTGTGTTCGTCA

                760        770        780        790        800
727 ACCGCAACAGCGTTTATAACAGCACCCTCATCAACTACACCGTCCTCTTT
627 ACCACAACAGCGTTTATAACAGCACCCTCATCAAC-------GTCCTCTTT

                810        820        830        840        850
777 TACAACCACTTTTTTTTCATTTACCACTTCTTTCTCTTTAACAAATTCA
671 TACAATCACTTTTTTTTCATTTACCACTTCTTTCTCTTTAACAAATTCA

                860        870        880        890        900
827 ACACACTGCCCAACTGCTCTTGTAAATTGTTTAAACGTTCATCAATGCTG
721 ACACACTACCCAACTGCTCTTGTAAATTGTTTAAACGTTCATCAATGCTG

                910        920        930        940        950
877 TTGCTCTTGTTGTCTTCGTCCAATTTCCTTTTGAGTTCATTCTCCACACC
771 TTGCTCTTGTTGTCTTCGTCCAATTTCCTTTTGAGTTCATTCTCCACACC

                960        970        980        990       1000
927 CACCACTCGTCTATATGTGACGGCGATATCTTGTTGGGTTTTGATGATTT
821 CACCACTCGTCTATATGTGACGGCGATATCTTGTTGGGTTTTGATGATTT

               1010       1020   <1030 ORF repeats   1040       1050
977 CATGGTGCAACTCTTTTAAAGAGGACATGTCTAAATATTAAATAGTCTTA
871 CATGGTGCAACTCTTTTAAAGAGGACATGTCC AAATATTAAATAGTCTTA

               1060       1070       1080       1090       1100
1027 TTAATAATGAGCTGGTATGATTTATTTTAATGTAACAGTTTATAACTTTA
921 TTAATAATGAGCTGGTAT T----------------------------

               1110       1120       1130       1140       1150
1077 ACATACAGTACAACATTTTATCTAATAGAGCGTGATGATGATCACCACCGTCT
939 ----------------------------------------------------

               1160 ORF(un1) >1170    1180       1190       1200
1127 TGGCATCCGGAAACCATGTTCATAACAGAACACGGGATTTGCGATCTAGT
939 ----------------------------------------------------

               1210       1220       1230       1240       1250
1177 AGCAGACAAACCTGAGGCCAAATGGAAGATGATAAAATTCATCTATAATA
939 ----------------------------------------------------

               1260       1270       1280       1290       1300
1227 AACTCTCTCCGAAAAGACAATGGCCTCAGTTTCAACCGAGACCGAGGCTG
939 ----------------------------------------------------

               1310       1320       1330 * ORF (un1) 1340       1350
1277 TTATTACCGCAGAAAGAACCTAAACCGCCGTAAAGAGAATCTTCTCCCCC
939 ----------------------------------------------------

               1360       1370       1380       1390       1400
1327 TCCACAACTCTCTACCCCCGAATATGGAGACGAACGTTCCGTACTCGTTT
939 ----------------------------------------------------

               1410       1420       1430       1440       1450
1377 ACGAATGTGACTATGAACTCGATCATTGATCTCATAATAAATTTCAGCAA
939 ----------------------------------------------------

               1460       1470       1480       1490       1500
1427 ACCTATCATAGGGTGATGATATGTATCGAGATGTCTAAAATAAAAGTTTT
939 ----------------------------------------------------
```

```
                1510        1520        1530        1540        1550
1477 ATTTCTATGTTTTATTTCCTATTCCTAGACATTCCTATAGAAAAAAAAAAC
939  ----------------------------------------------------

                1560        1570        1580        1590        1600
1527 AAAGACATATGACATTTTTTATAAATATAAAAGACATGTGAATCAATACA
939  ----------------------------------------------------

                1610        1620        1630        1640        1650
1577 TATCGAAACAGTCGCAATAATGATGACGATATTCGATATCTTCCGCGTAC
939  ----------------------------------------------------

                1660        1670        1680        1690        1700
1627 GGTGTGGTCGGTGAGTCGAGTCACTGTGCAAGTTCCTTTGTTTTTGTTAT
939  ----------------------------------------------------

                1710        1720        1730        1740        1750
1677 CTATCCTCTCTGCTTTGTTCTATCAAACCTCTTTATCAATCCTTTGTTCT
939  ----------------------------------------------------

                1760        1770        1780        1790        1800
1727 TTGTTATCACCTTCGAGAACCTATCTACTATGATTATTTGATTAGTTTAT
939  ----------------------------------------------------

                1810        1820        1830        1840        1850
1777 ATCTTTGGTATATAACGGAATACAACTCTGTAATCATCATTTATTTTCTT
939  ----------------------------------------------------

                1860        1870        1880        1890        1900
1827 TCGGCGTGCAATCGGTAAGTGTTTTTATTTATTTTTTAGGCCTAGAAACT
939  ----------------------------------------------------

                1910        1920        1930        1940        1950
1877 CAAAGTATAATATTGTTTTTATTTATTTATTAAATTATTTTTCACTAGGC
939  ----------------------------------------------------

                1960        1970        1980        1990        2000
1927 CTATGAAATAAGGTAGAACTTGAAACTCAAAGTATAATATGTATTAGCAG
939  ----------------------------------------------------

                2010        2020        2030        2040        2050
1977 CCGTACAGTAATTTTTTATTTTTCATAGATAACGTGTCACTCAAGATGAA
939  ----------------------------------------------------

                2060        2070        2080        2090        2100
2027 TCAAGAAAGTTTGAATCCAGAAAGTTTATATACCATCGCGATTAAGAAGG
939  ----------------------------------------------------

                2110        2120        2130        2140        2150
2077 TGAAGAAACATCTATCTGATAAAACCTCAGACGGTCTCAAGAAATTATGT
939  ----------------------------------------------------

                2160        2170        2180        2190        2200
2127 CTCCCTTCAACTATTTCTTCAGATCTAGACACCATGTATGTAGAATTACC
939  ----------------------------------------------------

                2210        2220        2230        2240        2250
2177 TCCCGGTTGGTATTATTATCCCGAAACTGGTCAATGTGTAATCGATAAGA
940  ----------------------------------------------------

                2260        2270        2280        2290        2300
2227 CTCTCATTGATGCCGAGGTTCATAAAACGATTTGTGCTCAGTGGAAAAGT
940  ----------------------------------------------------
```

ORF 66
(XcGV)>

2310 2320 2330 2340 2350
2277 GATAATATTAATGATTATCATGATTGTATTAACTATTTAATTCATTGGGT
940 ----------

2360 2370 2380 2390 2400
2327 TGTCTGGGATCTCGATCTGGATGCACCTGTGGTGGATGACACCGAAGAGA
940 ----------

2410 2420 2430 2440 2450
2377 TATATATGAATACGCTATTGCTTCAACATCGTTTACTGTCTCTTTTACGC
940 ----------

2460 2470 2480 2490 2500
2427 TTAGGTGGTTCGGGAAGACATCCCTGGAAGACAATGGTACGGTCTGTAAG
940 ----------

2510 2520 2530 2540 2550
2477 TGAAAAACCTAGATGTAGACTTATTAAGTATATTAAATTGAAAACTGATT
940 ----------

2560 2570 2580 2590 2600
2527 TTGTGATTCCTCCTTGGTTAAAGGTAGTTAAGCGTAGTGAAATAGAAAAG
940 ----------

2610 2620 2630 2640 2650
2577 AAAATGTTGAGGCCAAACAAATATTGGAAATATTCTATGCTTAGTCGTAA
940 ----------

2660 2670 2680 2690 2700
2627 TGATCGTCGCCGTAAACGTAAATACGAACCCTGCCACGACCACGACCACG
940 ----------

2710 2720 2730 ORF 66 (XcGV) * 2740 2750
2677 ACGAGTACGAGGAAGCTAAACGTAGATATTGTATGTAGGAATTTTTGTAT
940 ----------

2760 2770 2780 2790 2800
2727 ATTATTTTTGTGTAATAAAAATATAATATAAGAATTATTTTTTATTTGTA
940 ----------

2810 2820 2830 ORF 75 (XcGV) * 2840 2850
2777 TCTCAATCCCCTTCACAAAGACCGTTGTGTAGGACTCATAGGTTGCGCAC
940 ----------

2860 2870 2880 2890 2900
2827 TGTAGGCTGACTTATAGGAATTTTAGGTTGTTCTGAAACTATACGTTCTT
940 ----------

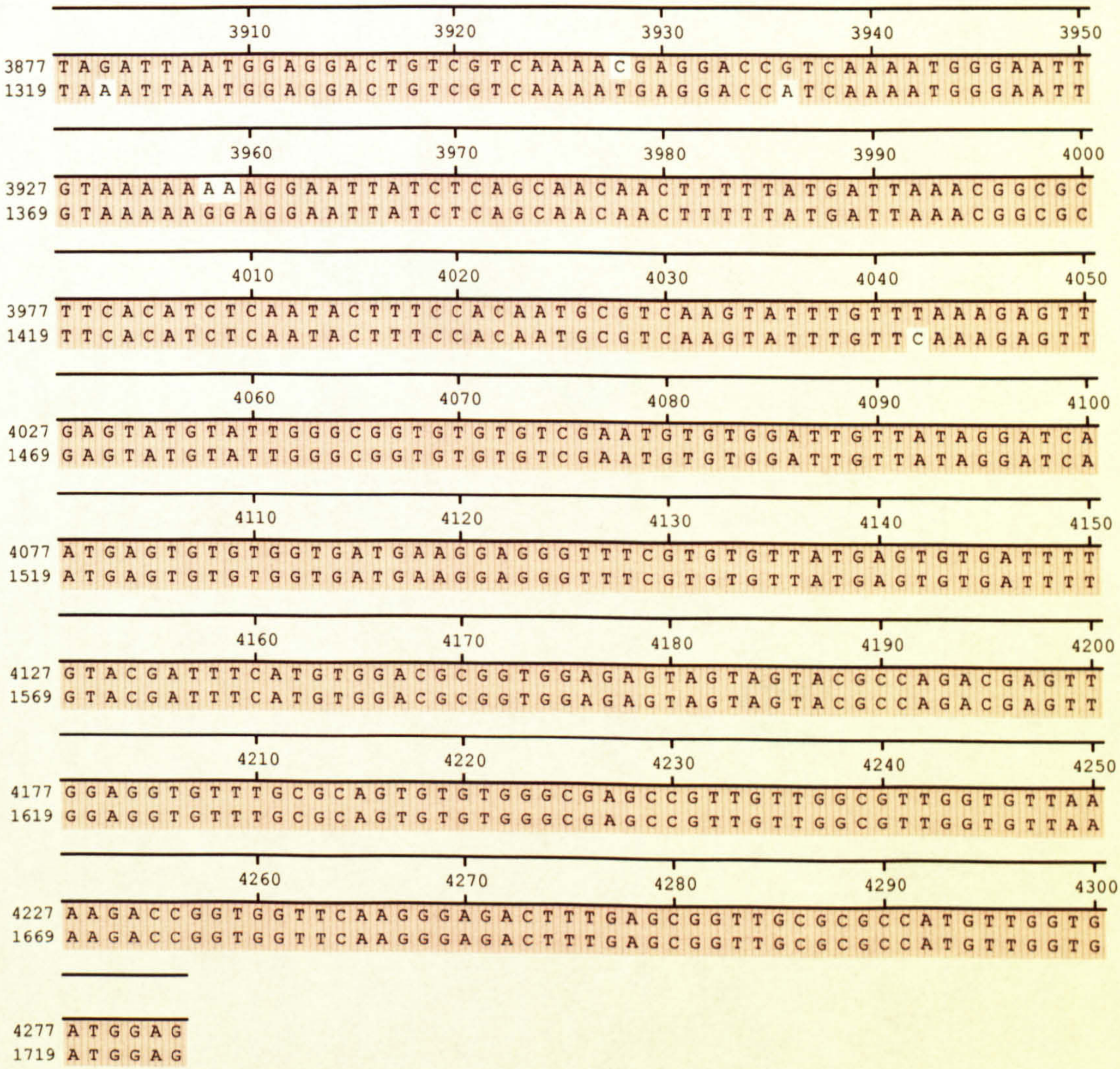
2910 2920 2930 2940 2950
2877 TCGCTGTTTTGCTTAGCTTCTTGATGCGCTTCTCTAACCTAACGACTTCC
940 ----------

2960 2970 2980 2990 3000
2927 CTCCTAGCACCGCGCCCTATGCAATGTGTCACGAACCTACGATGCACATT
940 ----------

3010 3020 3030 3040 3050
2977 ATTTGACATGCCCGTATAAAGAGCGCCGTCGGGTGTGCGCACCATGTAGA
940 ----------

3060 3070 ORF 75 <(XcGV) 3080 3090 3100
3027 CACACCAGCGTTGCGTTCCGATCATATTGTCTGTGTGTCTCTCTCTCTCT
940 ----------

```
                3110        3120        3130        3140        3150
3077 GTACTTACACATGTGACGACGGAATATTGCCCAGAACCACTTCCTACTAC
940  ----------------------------------------------------

                3160        3170        3180        3190        3200
3127 TCGAAGGTGCCTTGTCAAGACCCGCCAGAGTGCGAGATGCCGTTCAACAT
940  ----------------------------------------------------

                3210        3220        3230        3240        3250
                                        ptp >
3177 CATCTACAACTTCTTGAACCGTAACCTGAATGACAACAAAGGTCCTGGTG
940  ----------------------------------------------------

                3260        3270        3280        3290        3300
3227 CACTGTCACGCGGGCATTTCGAGATCTCCGACGGCGGTCATTTACTATTT
940  ----------------------------------------------------

                3310        3320        3330        3340        3350
3277 CATGCGTAAATACCAGATCGATTATGAAGCGGCGCTGCAGATGGTGAACC
940  ----------------------------------------------------

                3360        3370        3380        3390        3400
3327 GTAAGAGGAGAGTCGCACCCAGCGATCAATTTGTGGACATGTTGCGGAAC
940  ----------------------------------------------------

                3410        3420        3430        3440        3450
3377 AGATGTCATTATGTGTTTCGCGAAAACAAGAAGTGTGTGGTAGTTTTTTA
940  ----------------------------------------------------

ptp *           3460        3470        3480        3490        3500
3427 GGAGATGATGATAGGATAAAATAATAAAATATCATTTCATTATATAAACT
940  ----------------------------------------------------

                3510        3520 ORF(un2) *  3530        3540        3550
3477 TGTTTTATTTTATATTGATAAGAACTCACAAGACTACAGTGTCGTCGGAT
940  ----------GAACTCACAAGACTACAGTGTCGTCGGAT

                3560        3570        3580        3590        3600
3527 TGTATGGACCTAGAGCGGCTACGACGACGCACTGGTGATCTGGACCGTGG
969  TGTATGGACCTAGAGCGGCTACGACGACGCACTGGTGATCTGGACCGTGG

                3610        3620        3630        3640        3650
3577 TCGCGGCGAGTAATCTATGGACCTGCTGTGACTTCGTCTCTGTGGGCTGA
1019 TCGCGGCGAGTAATCTATGGACCTGCTGTGACTTCGTCTCTGTGGGCTGA

                3660        3670        3680        3690        3700
3627 CACGGCGGCGTGCACGGTACCTAGGATGCAGAATCGAGTTGGGGTGTTGG
1069 CACGGCGGCGTGCACGGTACCTAGGATGTAGAATCGAGTTGGGGTGTTGG

                3710        3720        3730        3740        3750
3677 TATGATTCGTCTTCCGAAGAGGAGGAGGATGACGACGATGATTCTGGATCTGG
1119 TATGATTCGTCTTCCGAAGAGGAGGAGGATGACGACGATGATTCTGGATCTGG

                3760 > p47  3770        3780        ORF(un2) < 3790        3800
3727 ACGGTTGGTAATGTCTCTGAGGGGTCGTCTTTTGGGCATGTTGTCTTATA
1169 ACGGTTGGTAATGTCTCTGAGGGGTCGTCTTTTGGGCATGCTGTCTTATA

                3810        3820        3830        3840        3850
3777 GAACGGTGGTCACAACGTGTAGGTTGCACTACACAATTACTTATATGAAT
1219 TAACGGTGGTCACAACGTGTAGGTTGCACCACACAATTACTTATATGAAT

                3860        3870        3880        3890        3900
3827 GTTTCGGCGGGTGTGTATGCACAGAGGATGGAATCTCAGCGGGAGTTTGA
1269 GTTTCGGCGGGTGTGTATGCACAGAGGATGGAATCTCAGCGGGAGTTTGA
```


3910 3920 3930 3940 3950
3877 TAGATTAATGGAGGACTGTCGTCAAAACGAGGACCGTCAAAATGGGAATT
1319 TAAATTAATGGAGGACTGTCGTCAAAATGAGGACCATCAAAATGGGAATT
3960 3970 3980 3990 4000
3927 GTAAAAAAAAAGGAATTATCTCAGCAACAACTTTTTATGATTAAACGGCGC
1369 GTAAAAAGGAGGAATTATCTCAGCAACAACTTTTTATGATTAAACGGCGC
4010 4020 4030 4040 4050
3977 TTCACATCTCAATACTTTCCACAATGCGTCAAGTATTTGTTTAAAGAGTT
1419 TTCACATCTCAATACTTTCCACAATGCGTCAAGTATTTGTTCAAAGAGTT
4060 4070 4080 4090 4100
4027 GAGTATGTATTGGGCGGTGTGTGTCGAATGTGTGGATTGTTATAGGATCA
1469 GAGTATGTATTGGGCGGTGTGTGTCGAATGTGTGGATTGTTATAGGATCA
4110 4120 4130 4140 4150
4077 ATGAGTGTGTGGTGATGAAGGAGGGTTTCGTGTGTTATGAGTGTGATTTT
1519 ATGAGTGTGTGGTGATGAAGGAGGGTTTCGTGTGTTATGAGTGTGATTTT
4160 4170 4180 4190 4200
4127 GTACGATTTCATGTGGACGCGGTGGAGAGTAGTAGTACGCCAGACGAGTT
1569 GTACGATTTCATGTGGACGCGGTGGAGAGTAGTAGTACGCCAGACGAGTT
4210 4220 4230 4240 4250
4177 GGAGGTGTTTGCGCAGTGTGTGGGCGAGCCGTTGTTGGCGTTGGTGTTAA
1619 GGAGGTGTTTGCGCAGTGTGTGGGCGAGCCGTTGTTGGCGTTGGTGTTAA
4260 4270 4280 4290 4300
4227 AAGACCGGTGGTTCAAGGGAGACTTTGAGCGGTTGCGCGCCATGTTGGTG
1669 AAGACCGGTGGTTCAAGGGAGACTTTGAGCGGTTGCGCGCCATGTTGGTG
4277 ATGGAG
1719 ATGGAG